IMPACT OF THE MATURATION STATUS OF OSTEOBLASTS ON THEIR HEMATOPOIETIC REGULATORY ACTIVITY

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ABSTRACT

Osteoblasts (OST) provide strong intrinsic growth modulatory activities on hematopoietic stem and progenitor cells via different mechanisms that include secretion of growth factors, and cellular interaction. Previously we showed that medium conditioned by mesenchymal stromal cell (MSC)-derived osteoblasts (M-OST) improve the expansion of cord blood (CB) CD34+ cells. I hypothesize that the hematopoietic supporting activity of M-OST would vary as a function of their maturation. This was tested by producing osteoblast conditioned media (OCM) from M-OST at distinct stages of maturation, and testing their growth regulatory activities in CB CD34+ cell cultures. My results showed that some of the growth promoting activity of OCM on CB cells are not dependent on the maturation status, while others are and those are largely independent of Notch signalling. In conclusion, these results provide further evidence that osteoblasts release factors that can promote the growth of immature CB progenitors in a Notch-independent way.

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ALP	Akaline phosphatase
Angpt-L	Angiopoietin-like proteins
AS- S	Alizarin Red S staining
BFU-E	Erythroid
BM	Bone marrow
BSP	Bone sialoprotein
CBT	CB transplantation
CFC	Colony forming cell assay
CFU	Colony forming unit
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
CM	Condition medium
OCM	Medium Conditioned with M-OST
CB	Cord blood
OMPC	Optimized megakaryocyte progenitor cocktail; SCF,TPO, FL
DLL1	Delta-like1
DLL3	Delta-like3
DLL4	Delta-like4
DMSO	Dimethyl sulfoxide
$CD235^{+}$ cells	Erythrocytes
E	Expanded
ECM	Extracellular matrix

ELDA	Extreme limiting dilution analysis
ETP	Earliest thymic progenitor
FBS	Fetal bovine serum
FGF-1	Fibroblast growth factor-1
FL	Flt3 ligand
FMO	Fluorescence minus one
G/M/GM	Granulocyte, monocyte/macrophage
GEMM	Granulocyte , Erythroid, Macrophage, Megakaryocyte
GMP	Granulocyte-monocyte progenitor
GvHD	Graft versus host disease
GFs	Growth factors
Hes1	Hairy Enhancer of Split
HSPC	Hematopoietic stem and progenitor cells
HSCs	Hematopoietic stem cells
HGF	Hepatocyte growth factor
Herp	HES-related Repressor Protein
IGFBP-2	Insulin-like growth factor binding protein 2
IL-3	Interleukin-3
IL-6	Interleukin-6
ISCT	International society for Cellular Therapy
ir MSC	Irradiated MSC
IMDM	Iscove’s modified Dulbecco medium
LDA	Limiting dilution

LSK	$Lin^{-}Sca1^{+}$ $cKit^{+}$
LTC-IC	Long-term culture initiating cell assay
LT-HSC	Long-term HSC
MEP	Megakaryocyte-erythroid progenitor
M-OST	MSC-derived osteoblasts
M-CSF	Macrophage colony-stimulating factor
$CD41^{+}$cells	Megakaryocyte
MK	Megakaryocyte
MSC	Mesenchymal stem cells
$CD14^{+}$ cells	Monocyte cells
MCM	Medium Conditioned with MSC
LMP	Immature lymphoid progenitor
MPP	Multipotent progenitors
NE	Non- expanded
NED	Notch extracellular domain
NID	Notch intracellular domain
NK	Natural killer
Ost-B10	OST derived from an MSC line HM3.B10
OSTs	Osteoblasts
OCN	Osteocalcin
OPN	Osteopontin
pNPP	P-nitrophenol phosphate
PPR	Parathyroid hormone/parathyroid hormone-related peptide Type 1 receptor

PB	Peripheral blood
e	Phenotypically like expanded
PBS	Phosphate-buffered saline
q-PCR	Quantitative PCR
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RT	Room temperature
SFM	Serum-free medium
ST-HSC	Short-term HSC
SCF	Stem cell factor
SR1	StemRegenin-1
TPO	Thrombopoietin
CFU	Total colony forming unit
TNC	Total nucleated cells
Col I	Type I collagen
α MEM	Alpha-minimal essential medium

CHAPTER 1 INTRODUCTION

The continuous process of blood-cells formation during development and throughout adulthood is called hematopoiesis, which produces and replenishes the blood and immune system. This is accomplished by maintaining a pool of hematopoietic stem cells (HSCs) that are capable of self-renewal and differentiation into all blood lineages (Fig. 1). In adults, HSCs reside primarily in the bone marrow (BM) before they mature and migrate into circulation (Shiozawa, Havens, Pienta, & Taichman, 2008). However, there are two other common sources of HSCs including mobilized peripheral blood (PB) stem cells, and cord blood (CB) (Geneugelijk & Spierings, 2015) that can be used in BM transplantation (Hidalgo & Frenette, 2005).

Under the classic model of hematopoiesis (Fig 1), HSCs can be partitioned into two groups; where long term HSC (LT-HSC) give rise to short-term (ST)-HSC (and more LT-HSC) and then multipotent progenitors (MPP). MPPs then split into two linages, the myeloid or lymphoid lineage and their respective common progenitor known as common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) (Sharpless & DePinho, 2007). These progenitors then undergo multiple stages of progenitor development toward various mature cell types such as leukocytes, erythrocytes, granulocytes etc. (Sharpless & DePinho, 2007). These properties, in combination with the ability of HSCs to engraft conditioned recipients upon transplantation, have facilitated stem cell use in regenerative medicine (Riddell et al., 2014). HSC transplantation has particular interest as a treatment option for diseases impairing a patient's ability to sustain or generate functional members of

the blood cell family. It is used in the treatment of congenital and acquired hematopoietic diseases, blood or bone marrow cancers such as leukemias, autoimmune diseases, and other malignancies (Riddell et al., 2014). The patient first has their immune system ablated, and then receives an HSC transplant, which hopefully replenishes their depleted hematopoietic stores with functional cell lineages. Depending on the condition treated, the source for HSC can be autologous (HSC from self) or allogenic (HSC from a donor).

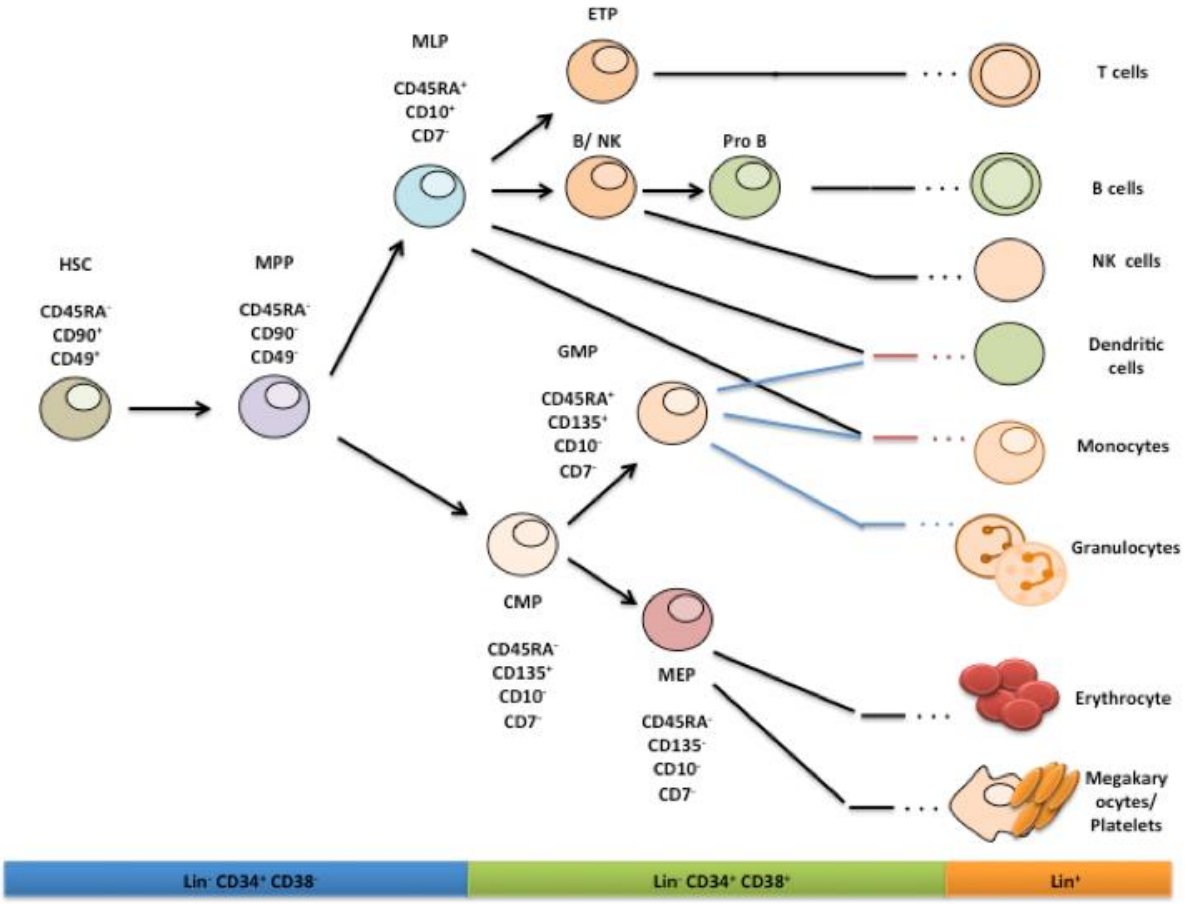


Figure 1. **Overview of the Current model of Human hematopoietic Hierarchy,** adapted from Doulatov, Notta, Laurenti, and Dick (2012)(Doulatov, Notta, Laurenti, & Dick, 2012). Immature lymphoid progenitor (MLP); Earliest thymic progenitor (ETP); Granulocyte-monocyte progenitor (GMP); Megakaryocyte-erythroid progenitor (MEP); Natural killer (NK). Lin: cocktail containing cell surface markers for all terminally differentiated populations (B cell; T cell; NK; dendritic cell, monocyte, granulocyte, megakaryocyte, and erythrocyte).

1.1 Cord blood transplantation:

CB is a great source of stem cells for allogeneic transplantation for patients who do not have a matched related or unrelated donor (Rocha et al., 2000). In 1988 the first CB transplantation (CBT) was performed successfully to cure an anaemic patient (Gluckman et al., 1989). CB is now considered a valid alternative to HSCs from BM and mobilized PB; especially effective for children due to the limited CB size (Tse & Laughlin, 2005). Typically, cells positive for cluster of differentiation 34 ($CD34^+$) are isolated from a CB unit which is understood to represent a population potent in HSPCs.

CB has some advantages over the other sources of HSCs due to its mode of collection and storage. The collection of CB is quite favorable, as it provides no risk to mother or baby and is far less invasive relative to BM or PB donations. Furthermore, by building CB banks we improve access to units of greater ethnic diversity (Geneugelijk & Spierings, 2015; Horwitz & Frassoni, 2015). Also, CBT have a reduced incidence and severity of Graft versus host disease (GvHD) (Rocha et al., 2000) and tolerance of higher degrees of HLA disparity between donor and recipient (Aldenhoven & Kurtzberg, 2015; Geneugelijk & Spierings, 2015). These benefits are believed to be a consequence of the naïve state of collected cells, and add significant value to the advantages CB transplantation.

On the other hand, CB also has some limitations. The principal issue with CB is the low dose of total nucleated cells (TNC) contained in a unit. Moreover, CB transplantation is also associated with delayed neutrophil and platelet recovery and higher rates of graft failure (Horwitz et al., 2014). Median time for neutrophil

engraftment is of 26 and 18 days after CB and BM transplants respectively. Median time for platelet engraftment is of 44 and 24 days after CB and BM transplants, respectively (Kellner, Li, Zweidler-McKay, Shpall, & McNiece, 2015). One method to overcome these restrictions is the use of two CB units, a double CBT; however this increases demand on vital resources and the risk for GvHD (Ballen, 2005; Geneugelijk & Spierings, 2015b; Horwitz & Frassoni, 2015). For these reasons and others, researchers have been developing various protocols to improve effectiveness of CBT.

1.1. 1 Ex vivo expansion of CB Stem and progenitors to improve engraftment

Many strategies have been developed to improve CB transplantation. A recent strategy shown to improve CB engraftment is an adaptation to the double CBT, where an unexpanded CB unit is co-transplanted with an *ex vivo* expanded unit (Fig. 2). The rationale is to accelerate the engraftment by transplanting a larger dose of progenitors produced *ex vivo* in HSC cultures. Delaney et al. (2010), using a Notch-based expansion protocol, reported the expanded unit was responsible for the improvement of neutrophil engraftment with a median time of only 16 days versus 26 days for conventional matched controls (Delaney et al., 2010). Meanwhile de Lima et al. (2012), using a MSC co-culture protocol, reported neutrophil engraftment within 15 days, and the cumulative incidence of neutrophil engraftment on day 26 was 88% versus 53–62% for controls. Moreover, platelet engraftment was also promoted and occurred within 42 days, with a cumulative incidence of engraftment on day 60 of 71% (expanded cells) versus 31–52% (control). Mixed chimerism post-transplant was evident in about half of the patients after 3–4 weeks

but long-term engraftment (12 months) originated predominantly from the unexpanded unit (de Lima et al., 2012). Accelerated neutrophil engraftment is important since it has been recently reported that this is associated, as expected, with decreased incidence of opportunistic infection in these patients (Summers, Milano, Gooley, Dahlberg, & Delaney, 2014).

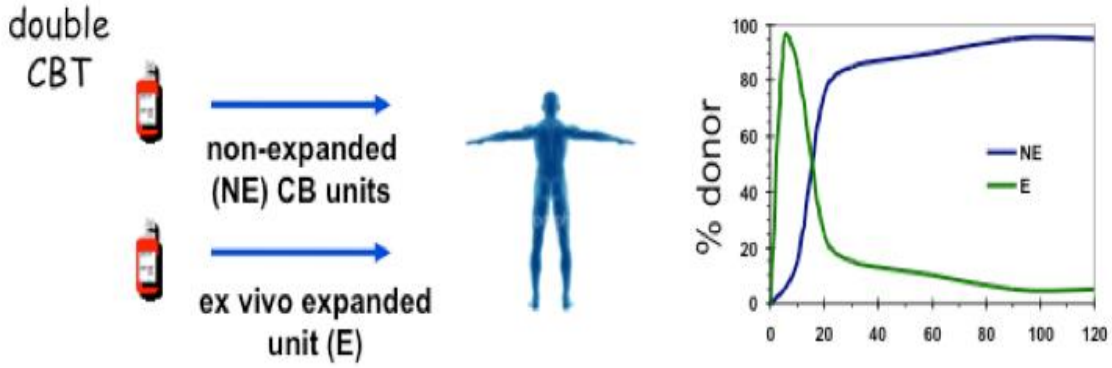


Figure 2. **<u>Double cord blood transplantation</u>,** a recent strategy where a non-expanded (NE) CB unit is co-transplanted with an *ex vivo* expanded unit (expansion allowing to increase cell dose). Promising results were obtained with double CB transplantation where the NE CB unit provides long-term engraftment while the E CB unit provides short-term engraftment.

Recently, Horwitz et al. (2014) reported the NiCcord study; co-transplantation of a non-expanded unit with an expanded CB unit with nicotinamide for 3-weeks co-infused with non-cultured T cells (cryopreserved) of the expanded units, accelerated neutrophil engraftment (with a median time 13 vs. 25 days, $P<0.001$) compared to historical control. Platelets engraftment was also achieved (with a median time 33 vs. 37 days, $P<0.085$) compared to historical control. More importantly, robust myeloid engraftment was achieved from the NIC-expanded unit in 8 out of 10 patients and was sustained more than 2 years (Horwitz et al., 2014).

More recently, in StemRegenin-1 (SR1) clinical trail, as a Stand-Alone Graft, Wanger et al. (2016) successfully achieved rapid neutrophil engraftment (11 days median time) in correlation to $CD34^+$ cell number. This strategy sustained durable myeloid engraftment for a median time follow-up, 272 days (Wagner et al., 2016). Hence, these studies and others have shown that *ex vivo* expansion of CB HSC and progenitors can make a significant contribution to engraftment.

1.2 Bone marrow microenvironment

In 1978, Schofield was the first scientist who claimed the existence of microenvironments known as niche in the BM where HSC reside (Schofield, 1978). These stem cell niches are critical for HSC maintenance, quiescence and functional activity. BM microenvironment contains many different cellular and soluble components that are essential for hematopoiesis, such as several stromal cells, cytokines, and chemokines. These provide distinct instructions to the cells via chemical signals and physical interactions which transduce the cells via various cell

signaling pathways (Mendelson & Frenette, 2014). MSC and OSTs are two important cellular components of the BM HSC niche that provide direct and indirect influences on HSCs, and will be discussed in this project.

1.2.1 Mesenchymal stem cells

MSC are multipotent stromal cells that represent an important cellular component of the BM microenvironment. The International society for Cellular Therapy (ISCT) defined MSCs to positively express CD73, CD90, CD105 and recently CD146 and CD166, while lacking expression of CD11b, CD14, CD34, CD45, CD19, CD79α and HLA-DR (Dominici et al., 2006). MSCs are capable of differentiating into numerous mesenchymal lineages such as chondrocytes, fibroblasts, adipocytes or osteoblasts depending on the activated signaling pathways (Birmingham et al., 2012).

MSCs have many phenotypes and properties that characterize them from any others cell types (Fig. 3). In culture, MSCs are plastic adherent and are capable of self-renewal and differentiate *in vitro* and *in vivo* to a series of specialized cells. *In vitro*, MSCs can be differentiated to OSTs by using standard osteogenic medium, which contains dexamethasone, ascorbic acid and β-glycerophosphate (Birmingham et al., 2012). OSts derived from MSC will be referred as M-OST in this project from now on.

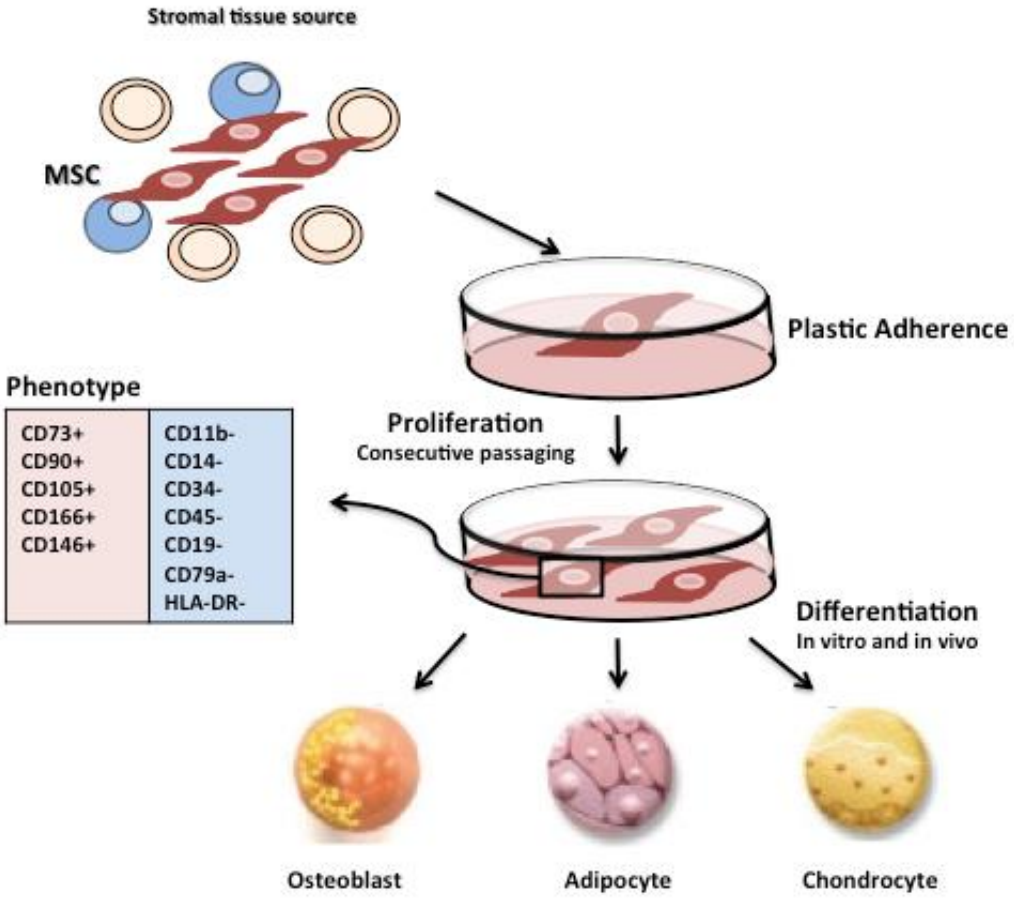


Figure 3. <u>Properties and phenotypes of MSCs</u> as adapted from Le Blanc et al. (2012) (Le Blanc & Mougiakakos, 2012).

MSCs are recognized as contributors to the HSC niche. They are capable of promoting HSC quiescence, maintenance, growth, or even differentiation mediated through the release of a wide array of cytokines, chemokines, growth factors (GFs), or even cell-to-cell contact (Dazzi, Ramasamy, Glennie, Jones, & Roberts, 2006; Devine & Hoffman, 2000). Several studies have shown that an *ex vivo* co-culture with MSC can improve engraftment of CB transplantation. Fei et al. (2007), using a human BM-MSC co-culture protocol, reported that MSC improve the expansion of CB $CD34^+$ cells and accelerate the ST-HSC reconstitution of expanded CB cells in NOD/SCID mice (Fei et al., 2007). Furthermore, Walenda et al. (2011) determined that MSCs are capable of supporting expansion of hematopoietic stem and progenitor cells (HSPC) in culture which was significantly elevated in the presence of a cytokine cocktail consisting of stem cell factor (SCF), thrombopoietin (TPO) and fibroblast growth factor-1 (FGF-1) (Walenda et al., 2011). Thus, MSC can promote the expansion and engrafting of CB HSPC likely through cell-to-cell contact and through the release of paracrine factors. These studies were supported by the clinical trial of de Lima et al. (2012). Transplanting expanded CB units reduced neutrophil engraftment to 15 days from 24 days for the un-manipulated units ($p<0.001$), while the median time for platelet engraftment also reduced to 42 days from 49 days ($p<0.03$), as previously mentioned in section 1.1. 1. (de Lima et al., 2012).

1.2.2 Osteoblast

OSTs are cells lining the endosteal surface of bones and they are responsible for the bone synthesis, remodeling and mineralization (Barros et al., 2010; Huang,

Yang, Shao, & Li, 2007). These processes are achieved by the ability of OSTs to secrete and express several proteins and genes such as type I collagen (Col I), Calcium, bone sialoprotein (BSP), osteocalcin (OCN), and osteopontin (OPN) that participate in the formation and mineralization of the bone extracellular matrix (ECM) (Huang et al., 2007). There are three main phases of OSTs differentiation (osteogensis): proliferation, matrix maturation, and mineralization. These stages can be characterized by the expression of unique OST markers including alkaline phosphatase (ALP), Col I, OPN (Born, Lischer, & Maniura-Weber, 2012), BSP, OCN, and parathyroid hormone/parathyroid hormone-related peptide Type 1 receptor (PPR), which are commonly used as OST markers (Huang et al., 2007). Moreover, many well-established methods such as immunohistochemical staining, Alizarin Red S (AR- S) calcium staining, and quantitative RT-PCR of osteogenic genes (for example the transcription factor *Runx2*, and *OPN*) have been used to monitor OST differentiation (Born et al., 2012).

OSTs are derived from MSCs and constitute an essential cellular component of the stromal cell support system in BM microenvironment for hematopoiesis (Jung et al., 2005). HSCs are found in close proximity to the endosteal surface of the trabecular bone which makes them intrinsically linked to the BM (Huang et al., 2007). Similarly to MSC, OST are capable of regulating hematopoiesis through the release of cytokines, chemokines or through cell-to-cell contact (Jung et al., 2005; Mishima et al., 2010). It has been suggested that OSTs produces several factors such as angiopoietin-1, CXCL12, OPN, macrophage colony-stimulating factor (M-CSF), SCF, Fit3 ligand (FL) and TPO that modulate HSC fate, quiescence, self-renewal and expansion *in vivo* or *ex vivo* (Barros et al., 2010; Mishima et al., 2010).

Many studies have provided evidence that OSTs are crucial cellular component of the HSC BM niche because of their capacity to effectively enhance HSC function and expansion by producing TPO and interleukin-3 (IL-3) GFs, and CXCL12 chemokine (Mishima et al., 2010). These expressed components are important for expansion and support of hematopoiesis in long-term cultures, suggesting that OSTs may provide an excellent *ex vivo* environment for hematopoiesis during progenitor cell expansion and may be important for *in vivo* cell therapy (Mishima et al., 2010). Interestingly, Calvi et al. showed a positive correlation between OST numbers and HSC numbers in which parathyroid hormone receptor 1 activation increased the expansion of OSTs, which in turn increased HSCs number, and vice versa (Laura M. Calvi & Link, 2015). As well, the collaboration of HSC and OSTs mediates the synthesis of several OST-derived cytokines to enhance HSC survival, such as interleukin-6 (IL-6) and hepatocyte growth factor (HGF).

The regulatory function of OSTs to promote ex vivo expansion of HSPC has been investigated by some co-culture studies. Ahmed et al. using human primary OSTs showed that these cells effectively facilitated the growth of BM $CD34^+$ cells with or without exogenous cytokines (Ahmed, Khokher, & Hassan, 1999). Research done with primary murine OST by Cheng et al. showed that immature OST support the expansion of HSC better than more mature OST. They used OSTs at different stages of maturation in a co-culture with Lin^-Sca1^+ $cKit^+$ (LSK) cells to investigate OST maturation consequence on modulation of HSPC growth. Freshly isolated OST presumably represented a more immature OST state, while pre-culturing OST for 1-3 weeks prior to experiment presumably represented a more mature cell. They found a significant increase (up to 3.4-fold) in the total HSPC expansion in cultures

of fresh OSTs compared to those containing OSTs cultured for 1-3 weeks. Moreover, the total colony forming unit (CFU) number and percentage of LSK cells was also significantly increased. Importantly, these results were corroborated by *in vivo* results in which fresh/immature OSTs enhanced the hematopoietic supporting capability more than cultured/mature OSTs (Cheng et al., 2011). These findings are also supported by the work of Chitteti et al. in which primary murine OSTs were co-cultured with LSK cells. They found that immature OSTs support greater expansion of immature HSPC than do mature OSTs. That showed an association between high level of *Runx2* expression and less mature OSTs, which was suggested to in-turn support modulation of hematopoiesis (Chitteti, Cheng, Streicher, et al., 2010). Interestingly, Mishima et al. (2010) demonstrated the ability of human Ost-B10 (OSTs derived from an MSC line HM3.B10) to regulate the growth of human HSPCs in co-culture setting. Using long-term culture initiating cell (LTC-IC) and colony forming cell (CFC) assays they found Ost-B10 support the expansion of multipotent hematopoietic progenitors of HSC (Mishima et al., 2010). More studies needed to determine what properties are common in between human and murine OST.

Numerous signaling systems are recognized to play important roles during OSTs development, which also contribute in their function to enhance HSC expansion, such as the Notch signaling pathway as describe below.

1.3 The Notch signalling pathway

The Notch signalling pathway is a highly conserved pathway that plays essential roles in various cellular functions such as cellular proliferation,

differentiation, and fate decision during homeostasis in human (Deng et al., 2008; Weber & Calvi, 2010). There are four notch receptors (Notch 1, 2, 3, and 4) and five notch ligands (Delta-like1 (DLL-1), Delta-like3 (DLL-3), Delta-like4 (DLL-4), Jagged 1 and Jagged 2) have been identified in mammals (Deng et al., 2008; Saito et al., 2014). It is important to note that the Notch receptor is a transmembrane protein that can be found on various cell types such as HSCs (Suresh & Irvine, 2015) .

The mechanism in which the Notch signaling pathway is activated can be summarized in the following steps depicted in Figure 4 (adapted from Andersson and Lendall, 2014). First, in order to activate the Notch signaling pathway, the Notch ligand in one cell needs to be in contact with the extracellular domain of the Notch receptor in a neighboring cell. This will cause two proteolytic cleavage reactions (extracellular & intracellular) at the Notch receptor. The Notch intracellular domain (NID) is activated by cleavage of the Notch extracellular domain (NED) by ADAM metalloprotease. Afterwards, the γ-secratase enzyme complex cleaves the Notch receptor at the site where it is anchored to the plasma membrane and releases the activated NID to initiate the signal (Suresh & Irvine, 2015). Afterward, the NID is translocated to the nucleus and modifies transcription to express many downstream target genes (Saito et al., 2014) such as Hairy Enhancer of Split (*Hes1*) (Weber & Calvi, 2010)2nd page and HES-related Repressor Protein (*Herp*) in mammals (Deng et al., 2008).

Notch regulates HSC and progenitor self-renewal and differentiation in various organs, such as in neural tissue, muscle, skin, and gut (Maillard et al., 2008). In the hematopoietic system, Notch is essential for the emergence of definitive HSCs during fetal life (Maillard et al., 2008). In addition, it has been widely

hypothesized that Notch signaling pathway is a critical component of HSC niches and promote HSC maintenance by activating Notch receptors expressed on HSCs. It was suggested that Notch signaling increases self-renewal and decreases differentiation of hematopoietic progenitors in LSK cell type (Weber & Calvi, 2010). Furthermore, Delaney et al. (2010) reported that when Notch ligand was infused in a CB CD34+ cell culture *ex vivo*, more than 100- fold increase in CD34+ cells was observed as well as improved engraftment activity (Delaney et al., 2010). Conversely, Benveniste et al reported that Notch is dispensable for *in vivo* maintenance of HSC, but was shown important for *ex vivo* expansion human CB HSC. This may be a result of the complex nature of the signaling environment in both systems (Benveniste et al., 2014).

Notch signaling has also been implicated in mediating the regulatory effect of OST on HSC. This was first revealed by the work of Calvi et al. (L. M. Calvi et al., 2003) in a mice model study. They demonstrated that the increase in the number HSC pool was a result of the activation of Notch signaling pathway through the interaction between HSC and OST (L. M. Calvi et al., 2003). Moreover, Chitteti et al. reported that CB TNC and CFC net output were significantly reduced when OST co-cultured with LSK in the presence of Notch inhibitor (GSI RO4929097). This provide evidence that Notch signalling was partially involved in OSTs function to promote murine HSC-enriched cells (Chitteti, Cheng, Poteat, et al., 2010).

Taken together, the Notch pathway has been implicated in the regulation of HSC in both ex vivo expansion culture system, and also in OST-based coculture system.

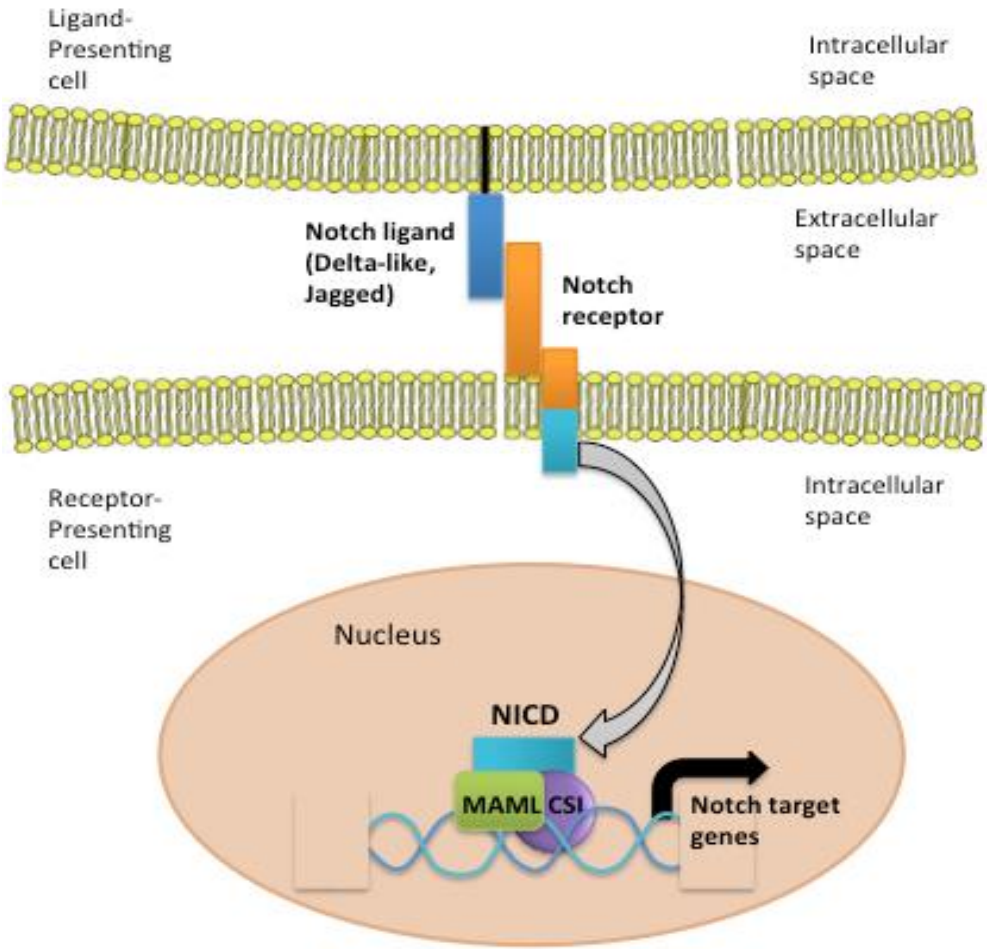


Figure 4. <u>Mechanism of Notch signaling pathway</u>, adapted from Andersson & Lendahl (2014) (Andersson & Lendahl, 2014).

1.4 Previous work in the Pineault laboratory

Dr. Pineault's lab found that expansion of megakaryocyte (MK) progenitors and CB CD34+ cells had reached a plateau in serum-free medium (SFM) supplemented with the cytokine cocktail OMPC (optimized megakaryocyte progenitor cocktail; SCF,TPO, FL), (Pineault et al., 2011). Hence, they went on to show that the *ex vivo* expansion of HSPCs using irradiated MSC (ir MSC) in a non-contact co-culture setting generally improved compared to SFM. This was in part due to the differentiation of ir MSC into OST-like cells (Celebi, Mantovani, & Pineault, 2011). Afterward in 2014, Pineault's lab demonstrated that medium conditioned with M-OST (i.e. OCM) was superior in enhancing the expansion of CB $CD34^+$ cells and myeloid progenitors relative to SFM or MSC-conditioned SFM (MCM) (Dumont et al., 2014). Most importantly, they found significant increases in the levels of human platelets when expanded cells were transplanted into mice compared to CB cells expanded in SFM or MCM (Dumont et al., 2014). As a result, the platelet recovery was more robust which is of considerable interest given the significant delay in platelet recovery associated with CB transplantation (Dumont et al., 2014) .

The results of the study by Dumont et al. (2014) raise several new questions and unresolved issues when I joined the laboratory. For one, the impact of OST maturation on the capacity of OCM to support the *ex vivo* expansion of HSPC was not well characterized and in second, the mechanism of action of OCM on CB progenitors was poorly understood. These two outstanding questions were an integral part of my thesis project presented herein.

1.5 Use of MSC-derived OST to study osteoblasts

The capacity of MSC to differentiate to several cell types include OST that provides a potential development in cell therapy. In vitro, the osteogenic differentiation of MSC can be achieved using standard osteogenic medium as mentioned before in section 1.2.1, (Birmingham et al., 2012). Throughout the process of differentiation and maturation of MSC, it has been shown that many morphological changes (Friedman, Long, & Hankenson, 2006; Kulterer et al., 2007) and standard OST-specific gene expression (Kulterer et al., 2007; Zhu, Friedman, Luo, Woolf, & Hankenson, 2012) were found associated with the process. Many studies used primary MSC and confirmed their differentiation potential to OSTs by AR-S staining, Alkaline Phosphatase (ALP) assay and gene expression of some OST-specific genes (Birmingham et al., 2012; Kulterer et al., 2007). Moreover, several OST-specific marker genes found to be unregulated during the differentiation process including *ALP, Col I, OPN, OCN and Runx2* (Born et al., 2012; Kulterer et al., 2007). These OST-specific genes were accompanied to the three main phases of osteogenic development mentioned previously in section 1.2.2 (Born et al., 2012). Interestingly, Kulterer et al (2007) found that MSC produced after seven days of differntiation using osteogenic medium was similar to primary human OST (Kulterer et al., 2007).

1.6 Hypothesis and research objectives

Our laboratory has previously shown that SFM conditioned with M-OST (i.e. OCM) can significantly promote expansion of CB $CD34^+$ cells in culture. The primary objective of my research project was to further elucidate properties of OCM which

modulate $CD34^+$ cell expansion, by focusing on how the differentiation and maturation stage of OST modulated the activity of OCM.

The driving hypothesis of my thesis is that the hematopoietic supporting activity of OCM will vary as a function of the M-OST differentiation and maturation status.

The following objectives were set to test my hypothesis.

Objective 1: Produce M-OST at distinct stages of maturation.

In order to test my hypothesis we first investigated the production M-OST at distinct stages of maturation to confirm that these differentiated OSTs from MSC can be used as an equivalent to primary OST, which would provide a simpler method for collecting OCM. This was done by varying the length of culture of MSC into osteogenic medium. At selected time points, the differentiation and maturation status of M-OST was investigated using complementary assays. OST differentiation will be investigated by quantitative-PCR (q-PCR) tracking the expression of Runx2 and osteocalcin. Next, calcium deposits will be visualized by Al red stain and quantified by elution of the stain. Finally, ALP activity in OST will be measure using ALP kit.

Objective 2: Quantify the expansion of CB cells in CD34+ cell cultures done with OCM prepared with M-OST at distinct stages of maturation

To achieve this objective, I first set to characterize the hematopoietic supporting activity of MCM and OCMs for the growth of CB CD34+ cells in cultures. The impact of the CM on expansion of CD34+ cells and their differentiation was assessed by flow cytometry analysis. Moreover, for selected cultures, we also tested

the capacity of CM to support the production of committed progenitors and multipotent progenitors using the CFCs assay and the LTC-IC assay, respectively.

Objective 3: Test the Implication of Notch signaling in the mediation of OCM activity on CB CD34^{+} cell growth promotion.

First, I investigated the expression pattern of four Notch ligands in MSC and M-OST. Then, I tested whether the pro expansion activity of OCM on CB cell growth, CD34+ cells and progenitors was impacted or not by inhibition of notch signaling using a gama-secretase inhibitor.

Objective 4: Compare the gene expression pattern of selected growth factors of HSPC in MSC and M-OST

I investigated the expression of selected genes from angiopoietin-like protein (*Angpt-L)* family members and insulin-like growth factor binding protein-2 (*IGFBP-2)* by q-PCR.

CHAPTER 2 MATERIALS AND METHODS

2.1 Isolation and culture of human mesenchymal stromal cells

Fresh human BM MNCs (Lonza, Group Ltd, Basel, Switzerland) were cultured in alpha-minimal essential medium (α MEM) (Life Technologies, MA, USA) supplemented with 10% HyClone fetal bovine serum (FBS) (Fisher Scientific, Colonnade Rd, Ottawa, Canada), then incubated at 37°C in a humidified incubator (5% CO_2). On day three, cells in suspension were discarded and fresh medium added. At 85%– 90% confluence, adherent MSCs were trypsinized with TryplE Select 1X (Life Technologies), and cell viability was checked by Trypan Blue Stain 0.4% (Life Technologies). A total of five MSC lines were generated from independent samples. Cytometry analyses confirmed that MSCs were CD45-CD73+CD90+CD105+ and *in vitro* assays confirmed their capacity to undergo osteoblast, adipocyte, and chondrocyte differentiation (previously displayed in lab).

2.2 Production of MSC-derived osteoblasts

Osteogenic differentiation was induced by culturing confluent MSCs in osteogenic media (Jaiswal, Haynesworth, Caplan, & Bruder, 1997), which contains α-MEM supplemented with 10% FBS, 10^{-7} M Dexamethasone (Sigma, St-Louis, MO, USA), 0.17 mM ascorbic acid, and 10 mM β-glycerophosphate (Sigma), for 3, 6, 10, 14, and 21 days with medium replacement done every 2-3 days.

2.3 Production of MSC and M-OST Condition medium CM

Plates containing MSCs or M-OSTs were rinsed twice with phosphate-

buffered saline (PBS) and then incubated overnight with standard SFM consisting of Iscove's modified Dulbecco medium (IMDM) (Life technologies), insulin, and transferrin solution serum substitute BIT (StemCell Technologies, Vancouver, BC, Canada), 40 mg/ml of low-density lipoproteins LDL (Stem Cell Technologies), and 5 $x10^{-5}$ M β-mercaptoethanol (Sigma). CM collected from MSC and M-OST are referred as MCM and OCM respectively. CM produced 2 and 20 ml per 6- well and T-75 flasks, respectively. After incubation time, supernatants were collected and centrifuged (329xg/5 min at room temperature (RT)). All CMs were aliquoted and stored at -80°C until time of use.

2.4 Assessment of osteogenic differentiation and maturation

2.4.1 Alizarin Red-S Assay

AR- S staining (Sigma) is a dye used to evaluate calcium deposits in cell culture. After culturing MSC and differentiating them to M-OST for 3, 6, 10, 14, and 21 days using osteogenic media, cells were rinsed with phosphate-buffer saline (PBS) then fixed with ice-cold 70% ethanol for one hour at -20ºC, after which M-OST plates were left to warm up to RT and rinsed with dH_2O. Cells were then stained with 40mM of AR-S for 10min at RT on a shaker. Afterward culture was rinsed with dH_2O followed by PBS (15 min). Microscopy pictures were taken in this stage using an Olympus CKX41 inverted microscope using 10X objective (Olympus Corporation, Japan). Next, 1% cetylpyridinium chloride CPC (Sima-Aldrich St. Louis, MO, USA) solution was used to extract AR-S (15 min, at RT, with shaking). Aliquots of the extract were diluted (1:5, 1:10, and 1:20) using the 1% CPC solution. Standards of AR-S were prepared in 1% CPC solution, starting with1:50 dilution then a series of

1:2 dilutions. A 1:50 dilution would bind 64.128mg of calcium given that AR- S binds 2 mol of calcium/mole of dye in solution (Stanford et al, Journal of Biological Chemistry, 1995). AR-S concentration was determined by measurement at 620 nm using a microplate reader (Expert Plus, Montreal Biotech, Montreal, Quebec, Canada). A standard curve composed of the 1:2 dilution series of the same solution was used to standardize the AR- S staining. Extracellular calcium deposits in differentiated OSTs should be colored bright orange-red with the stain.

2.4.2 Alkaline phosphatase assay

MSCs were plated into 35mm dishes for OST differentiation. At each time point, cells were washed with PBS, trypsinized, pelleted and suspended in 150 µl of Assay Buffer (abcam, 1 Kendall Square, Cambridge, MA, USA). Next, they were vortexed and transferred to a microtube, then centrifuged at 13,000xg for 3 min at 4°C. Afterward, the supernatant from each tube was transferred into a new tube and stored at -80°C until all samples were ready to be tested with the ALP kit (ab83369, abcam). Manufacturer's instructions were followed to perform the assay. Briefly, the colorimetric conversion of p-nitrophenol phosphate (pNPP) to p-nitrophenol was used to determine ALP activity. Different dilutions of cell lysates were incubated with 5 mM of pNPP for 60 min at 25°C in the dark. Next, the reaction of ALP was terminated by adding 20µl of Stop solution to all the samples. Finally, the absorbance was measured at 405nm using a microplate reader and the ALP activity was determined by comparison with the use of 0, 0.004, 0.008, 0.012, 0.016 and 0.020 µmol of pNPP for standard curve.

2.5 CD34^{+} cell isolation

The Canadian Blood Services' Cord blood for Research Program provided all of the units of human umbilical CB used in this study after institutional review board approval and written informed consent from the mothers. The isolation of MNC was performed using Ficoll-Paque PLUS (GE Health Care Inc., Baie D'Urfe, Canada). CB CD34+ cells were isolated using magnetic beads via the EasySep ™ Human Cord Blood CD34 Positive selection kit (Stem Cell Technology) according to the manufacturer's protocol. Typically, 2-3 units of CB were pooled for CD34+ cells enrichment and verified for ≥ 95% purity by flow cytometry using the Attune acoustic focusing flow cytometer (Blue-Red laser) (Applied Biosystems By Thermo Fisher Scientific, Waltham, MA, USA) with CD34- PE (518) antibody (Becton Dickinson Pharmingen, Mississauga, Canada).

2.6 Culture of CD34^{+} cells

CB CD34+ cells (7,500-20,000 cells/mL) were cultured as indicated for 6 days. Conditions used in this set of experiments were: SFM control (Ctrl), MCM, Day 3 OCM, Day6 OCM, Day10 OCM, Day 14 OCM, and Day 21 OCM. All condition were supplemented with megakaryocyte progenitor cytokine cocktail OMPC, composed of 10 ng/mL SCF, 35 ng/mL TPO and 11 ng/mL FL, which were optimized for the expansion of megakaryocyte and myeloid progenitors (Pineault, 2011) (Pineault et al., 2011). All cytokines were purchased from Peprotech (Rocky Hill, NJ, USA). All conditions were in duplicate and started with one volume of each CM/well and incubated at 37^{o}C and 5% CO_2 in a humidified incubator. On the fourth day of culturing, one volume of each fresh CM was added. After six days of culture,

viable nucleated cells were counted manually with Trypan Blue Stain (Life Technologies) or by flow cytometry analysis, and analyzed using Attune acoustic focusing flow cytometer or FACS-Attune for surface phenotype expression of specific antigens, Results were repeated in at least 3 independent biological experiments with the use of different donor-derived MSC lines and donor-derived CB $CD34^+$ cell preparations unless stated.

In some cultures, different concentrations of a γ -secretase inhibitor, RO4929097 (Selleck chemicals, Houston, Texas, cat# S1575), were used to test if the Notch signalling pathway is implicated in the growth enhancing effect of OCM produced in this study. RO4929097 was reconstituted in DMSO and stored at -80^{o}C until required. Conditions used in this set of experiments were SFM vs OCM from day 6 M-OST, with each condition also have a vehicle control consisting of solely dimethyl sulfoxide DMSO addition without inhibitor. All conditions were supplemented with OMPC as mentioned above. Concentrations of inhibitor used were 2µM and 10µM, culture maintained for 4 days with fresh inhibitor added on every second day. Results were repeated in 5 independent biological experiments for flow cytometry analysis and 2 independent biological experiments for CFC, with the use of different donor-derived BM lines and donor-derived CB $CD34^+$ cell preparations.

2.7 Progenitors: colony forming cells assay (MethoCult)

CFC assay is a functional *in vitro* assay used to estimate the number of colony-forming progenitors in a sample to help check the potency of the HSPCs. After six days of culture of specified conditions, cells were counted using Trypan

Blue Stain and diluted into equal concentrations in IMDM and 2% FBS. Following manufacturer instructions cell suspensions were added to MethoCult M4434^{TM} Medium aliquots (StemCell Technologies) at a concentration of 400 cells/ml and vortexed at maximum speed for 15s to mix. Next, they were plated in 35mm dishes in duplicates and incubated for 14 days in a humidified incubator at 37 C and 5% CO_2. On day 14 colonies were scored using Olympus CKX41 inverted microscope. Scoring consisted of identifying and counting granulocyte/erythroid/monocyte/macrophage (GEMM), granulocyte/macrophage (G/M/GM) or blast forming unit erythroid (BFU-E) colonies depending on their morphology.

2.8 Long Term Culture – Initiating Cell assay

LTC-IC assay is an *in vitro* functional assay commonly used to measure hematopoietic multipotent progenitors. After six days of culture using OCMs, LTC-IC assay was performed in limiting dilution (LDA) setup by plating decreasing doses of CB cells (5,000, 1,000, and 250 cells), to determine the frequency of LTC-IC in the different conditions used in this study. The MS-5 stromal cell line was used as a feeder layer. 96- well plates coated with 0.1% gelatin (Stemcell Technologies) were plated with MS-5 using α-MEM medium with 10% FBS. The procedure was performed using the manufacturer protocol for human LTC-IC assay. Briefly, cells were suspended in long-term culture medium (MyeloCult M5300^{TM}, from StemCell Technologies) to which freshly dissolved hydrocortisone sodium hemisuccinate (Sigma) was added just prior to use to give a final concentration of 10^{-6} M.

Cultures were maintained for 5 weeks in humidified incubator at 37 C and 5% CO_2, with weekly half medium changes. After 5 weeks, the entire well content, including adherent and non-adherent cells, was transferred into individual methylcellulose cultures (MethoCult™ H4434) and plates were scored after 2 weeks. Dishes were scored by recording growth (≥ 1 colony) or no growth (no colonies). The frequency of LTC-IC were determined by Poisson statistics using the extreme limiting dilution analysis (ELDA) (Hu & Smyth, 2009).

2.9 Flow cytometry analysis

CB cells (100µl of well-mixed cell suspensions) were stained with monoclonal antibodies for 20 min at 4^{o}C in the dark using different panels of antibodies. Cells were prepared for analysis by addition of 900 µl of PBS and analyzed Flow cytometry using a FACS-Attune (Thermo Fisher, Waltham, USA). At least 1,00,000 events were acquired for each sample, with dead cells gated out by the forward and side-scatter and/or SytoxAAD staining (Life technologies). Appropriate fluorescence minus one (FMO) controls were prepared for each marker for multicolor analysis. The antibodies used included CD14- allophycocyanine (APC) (M5E2), CD235a-conjugated to phycoerythrine (PE) (GA-R2 (HIR2)), CD41a (GPIIb)-fluorescenceince isothiocyanate (FITC) (HIP8), CD34-PE (518), CD45RA-APC (HI100), CD38-FITC (HIT2), and CD90-PerCP-Cy7 (5E10), and Alkaline phosphatase-PerCP-Cy5.5 (B4-78). All antibodies purchased at Becton Dickinson Pharmingen (Mississauga, Canada). Cell count was performed simulatenously as the Attune provides a concentration for each sample which when multiplied by the final volume (after taking into account dilution factors) will produce the total cell

count. Expansion is a product of frequency and cell count. Samples were resuspended beforehand and not centrifuged to ensure a representative homogenous cell count reading from each sample. It was found in our lab that the coefficient of variation for this technique is 5%, which considered much better than manual count which was found to have a coefficient of variation of 10%.

2.10 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

RNA isolation of MSC and M-OSTs was performed using TRIzol® (Life Technology) following the manufacturer's protocol. RNA concentration and purity was determined using Bio Drop spetrophotometer (Montreal Biotech Inc., Kirkland, QC, Canada). iScript™ Reverse Transcription Supermix For RT-qPCR (BioRad, Hercules, CA, USA) was used for cDNA synthesis from 0.5-2mg of total RNA. Synthesis was performed using a thermal cycler (iCycler iQTM Real Time Detection System, BioRad, Hemel Hamstead, UK) and the following run protocol: 5 min at 25°C, 30 min at 42°C and finally 5 min at 85°C. Next, cDNA was diluted 1:5x or 1:25x for Q-PCR reactions using Sso Advanced Universal Sybr Green Supermix (BioRad) in a total volume of 10 µl containing 1 µl of diluted cDNA. All reactions were performed in triplicate using CFX96 TM Real-Time System (BioRad) following these steps: 95 ^{o}C/3 min, followed by 40 cycles of 95 ^{o}C/10 sec, 60^{o}C/20 sec, 72 ^{o}C/20 sec and melt curve analysis of 95 ^{o}C/10 sec, 65 ^{o}C/5 sec, 95^{o}C/5s. Data analyzed using the CFX Manager 3.1 software (BioRad) of the BioRad system and the standard curve method established by (Larionov, Krause, & Miller, 2005).

Table 1. PCR primer sequences using IDT Primer Quest (www.idtdna.com/SciTools).

Gene	Primer Pair	Sequence	Accession #
Angpt-L1	Forward Reverse	5'-CACCACACTGGACAGAGATAAA-3' 5'-CTTGGTGCTTGCTTCTGTAATG-3'	NM_004673.3
Angpt-L2	Forward Reverse	5'-CGGGATGCTGGATTCTGTT-3' 5'-GTCTGGTGTGAAGGAAAGTAGG-3'	NM_ 012098
Angpt-L3	Forward Reverse	5'-CCATAAGACGAAGGGCCAAA-3' 5'-CACTGGTTTGCAGCGATAGA-3'	NM_014495.3
Angpt-L5	Forward Reverse	5'-CATCCCAAGCCTCACTCTTATT-3' 5'-CTACTGAAGAGTCCGTAGAATGATG-3'	NM_178127.4
DLL4	Forward Reverse	5'-CCTCTCCAACTGCCCTTCAATTTC-3' 5'-ATGAGTGCATCTGGTGGCAAGG-3'	
GAPDH	Forward Reverse	5'-TCGACAGTCAGCCGCATCTTCTTT-3' 5'-ACCAAATCCGTTGACTCCGACCTT-3'	IDT Primer Quest
Hes 1	Forward Reverse	5'-AACACGACACCGGATAAAC-3' 5'-CCGCGAGCTATCTTTCTTC-3'	NM_005524
IGFBP2	Forward Reverse	5'-CGAGGGCACTTGTGAGAAGC-3' 5'-ATGTTCATGGTGCTGTCCACG-3'	X16302.1
Osteocalcin	Forward Reverse	5'-CCCAGGCGCTACCTGTATCAA-3' 5'-GGTCAGCCAACTCGTCACAGTC-3'	NM_199173.4
Runx2	Forward Reverse	5'-GATGGGACTGTGGTTACTGTCA-3' 5'-CTCAGATCGTTGAACCTTGC-3'	NM_001024630
BNSP (Osteopontin)	Forward Reverse	5'-CAGTTGTCCCCACAGTAGACAC-3' 5'-GTGATGTCCTCGTCTGTAGCATC-3'	J04765.1
Deltex	Forward Reverse	5'-GCCATGTACTCCAATGGCAACAAG-3' 5'-GGGGATGAGGTGGAACTCCATCTT-3'	NM_004416.2

Primers for DLL1, Jagged1, Jagged2 were ordered at Bio-Rad (Mississauga, Canada). Amplicons ranged in size from 94-117 bps for all primer designed in this study. The standard curve method was used for the relative quantification of real time PCR product (Larionov et al., 2005). PCR efficiency was assessed with a dilution series using pooled DNA from all samples, while melt curve analysis confirmed primer specificity.

2.11 Statistical analysis

Statistical analyses were performed using GraphPad InStat version 3.00 (La Jolla, CA, USA). Multigroup comparisons were done by paired ANOVA test (Dunnet Multiple comparisons test) while 2 groups comparisons were done with paired student-t-tests as indicated. We considered p values smaller than 0.05 to be significant.

CHAPTER 3 RESULTS

3.1 Production of M-OST at distinct stages of maturation

MSCs isolated form human BM were differentiated into M-OSTs using osteogenic differentiation medium using method established in (Jaiswal et al., 1997). To monitor the differentiation and maturation status of M-OST after distinct length of culture in osteogenic medium, three distinct assays were used; qPCR on osteogenic genes **(***Runx2 and Osteocalcin*), qualitative and quantitative analysis of calcium deposits by AR-S, and quantitative analysis of ALP activity.

First we tracked osteogenic differentiation and maturation by qPCR. The purpose was to test the expression of osteogenic genes to monitor the differentiation and maturation of MSC into M-OSTs. RNA was isolated then reverse transcribed into cDNA from undifferentiated MSC and M-OST at distinct culture time points. We chose to test the expression of *Runx2* and *Osteocalcin*. These two osteogenic genes are standard markers of OSTs differentiation and maturation, respectively (Born et al., 2012; Friedman et al., 2006). *Runx2* is a transcriptional factor key for osteogenic differentiation that correlate with their differentiation (Neve, Corrado, & Cantatore, 2011). OST maturation (signified by bone mineralization and presence of calcium deposits) is associated with their ability to express Osteocalcin, a non-collagenous protein (Neve et al., 2011).

For the qPCR results, gene expression was normalized to the housekeeping gene *GAPDH* then compared to that measured in undifferentiated MSC using the standard curve method established by (Larionov et al., 2005). Representative qPCR results obtained with 1 of 3 lines tested results show noticeable increases in the

expression of the osteogenic genes *Runx2 and Osteocalcin* in MSC undergoing osteogenic differentiation. This increase was apparent after just 3 days of culture in osteogenic medium for *Runx2*. qPCR analysis found that the expression of *Runx2* significantly increased by at least 4x in all M-OSTs compared to MSC, which was determined statistically significant in all M-OST samples ($p<0.001$, Fig. 5A). Next, I tested the expression of *Osteocalcin* which was significantly increased in all M-OST compared to MSC ($p<0.001$ vs. MSC, Fig 5B).

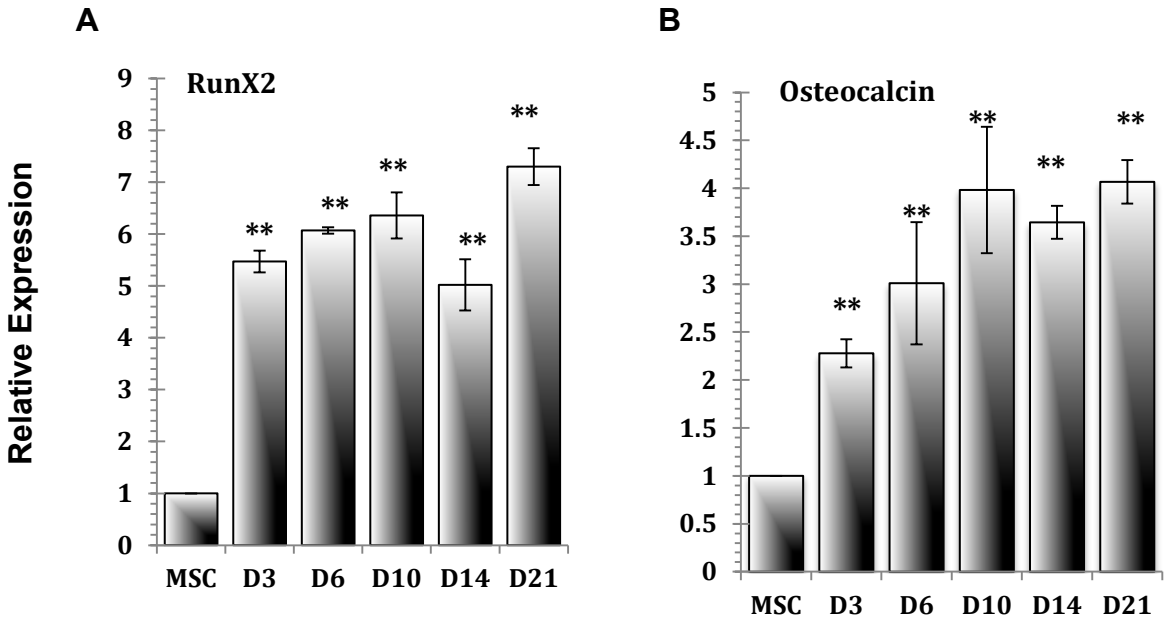


Figure 5. Expression of Osteogenic genes in MSC and M-OST as a function of culture time

qPCR results show noticeable increases in the expression of the osteogenic genes *RunX2* (A) and *Osteocalcin* (B) in M-OST compared to MSC. Relative expression normalized to *GAPDH*. Mean ± SD of 3 replicates (representative results from 1 MSC line shown, n=3). Data analyzed by paired ANOVA test (Dunnet Multiple comparisons test) *p <0.05, **p <0.01compared to Ctrl.

Next, I used AR-S to detect calcium deposits in M-OST cultures after distinct length of osteogenic cultures. Extracellular calcium deposits in M-OST are colored bright orange-red with this stain (Stanford, Jacobson, Eanes, Lembke, & Midura, 1995). Microscopy pictures were taken at each time point to monitor calcium deposit staining (Fig. 6A). The pictures showed very few if any calcium deposits in M-OST cultures before day 10 (Fig. 6A). However, after which as M-OST become more mature the number and size of the calcium deposits increased considerably. Day 21 M-OST showed the highest calcium deposits (Fig. 6A). Subsequently, calcium deposits were quantitated by eluting AR-S to determine the concentration of calcium in the deposit at each time point (n=2). It can be observed that calcium deposits significantly increased in M-OST as a function of their maturation time, with the highest level seen in day 21 M-OST cultures (Fig. 6B). These data confirmed the differentiation of MSC to M-OSTs and further maturation is positively correlated with length of culture in osteogenic medium.

Thirdly, I investigated the expression and activity of the enzyme ALP. This enzyme is produced by variety of cell types including OSTs (Sabokbar, Millett, Myer, & Rushton, 1994). ALP has long been used as an indicator of normal osteoblastic activity; this enzyme provides the alkaline environment to help calcium deposit to build bones (Golub & Boesze-Battaglia, 2007). This enzyme is expressed in the membrane of the OST and can be detected using an ALP antibody and analyzed by flow cytometry. Representative flow cytometry analysis of ALP in MSC and M-OST demonstrated that the frequency of ALP^+ cells and the mean fluorescent intensity of ALP protein increased over osteogenic culture time (Fig. 6C) suggesting increased

extracellular expression of ALP. Finally, I quantified the activity of ALP in MSC and M-OST at distinct stages of maturation using an ALP colorimetric assay (n=2). This kit uses pNPP as a phosphatase substrate that becomes yellow when dephosphorylated by ALP. It was observed that ALP activity increased in all M-OSTs compared to MSC, though differences were not quite significant (Fig.6D). Nonetheless, these results are consistent with the increase maturation over osteogenic culture time; ALP showed the highest activities in M-OST produced after 14 and 21 days of culture.

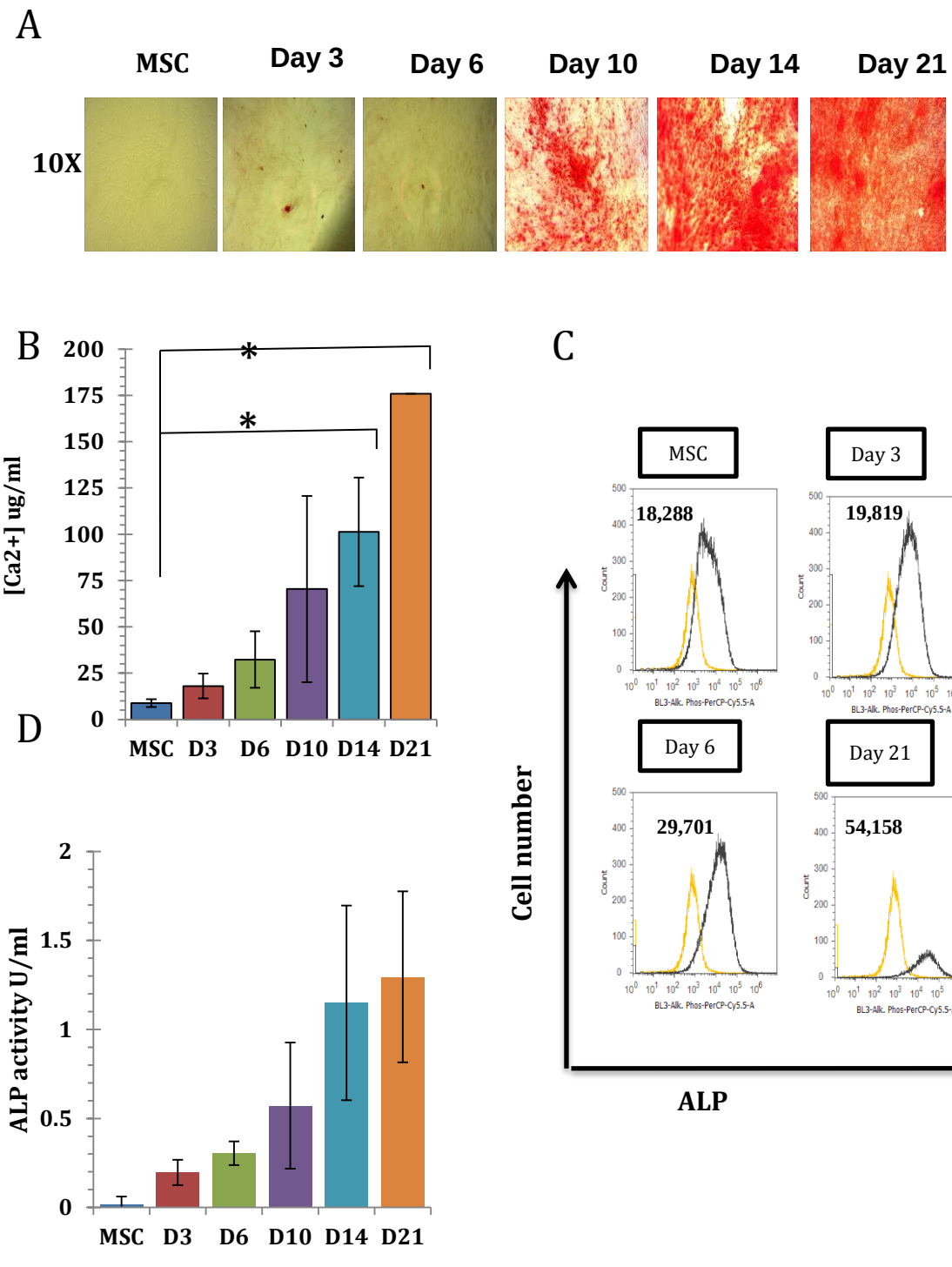

A
MSC
Day 3
Day 6
Day 10
Day 14
Day 21
10X
B
[Ca2+] ug/ml
200
175
150
125
100
75
50
25
0
MSC D3 D6 D10 D14 D21
C
MSC
Day 3
Day 6
Day 21
18,288
19,819
29,701
54,158
Count
BL3-Alk. Phos-PerCP-Cy5.5-A
Cell number
ALP
D
ALP activity U/ml
2
1.5
1
0.5
0
MSC D3 D6 D10 D14 D21

Figure 6. Osteogenic differentiation kinetics of human MSC

A) Detection of calcium deposits using AR-S in undifferentiated MSC and M-OST as a function of culture length (mean ± SEM of 2 independent cultures). B) Quantitative detection of calcium deposits for AR-S (mean ± SEM of 2 independent cultures). *p<0.05,**p<0.01 Data analyzed by ANOVA. C) Representative flow cytometry analysis of cell-surface ALP expression on undifferentiated MSC and M-OST at selected time points. The mean fluorescent intensity of ALP+ cells is shown for this representative experiment (n=3). D) ALP activity in undifferentiated MSC and M-OST as a function of culture length (mean ± SEM of 2 independent cultures).

In summary, these results are consistent with a rapid commitment and differentiation of MSC into M-OST and their progressive maturation into OSTs as a function of culture length. The variation in the expression of osteogenic genes, calcium deposit and ALP activity in the M-OSTs at different time points confirmed that we successfully generated OSTs at different maturation states. Based on qPCR results and functional properties of M-OST, we refereed to day 3 M-OST as pre-osteoblast, day 6-10 as immature M-OST and day 14-21 as mature M-OST (Friedman et al., 2006; Neve, Corrado, & Cantatore, 2011). These M-OST cultures were used to produce osteoblast-conditioned media (OCM) from OST at distinct stages of maturation.

3.2 Characterization of the hematopoietic supporting activity of M-OST

3.2.1 Production of serum-free media conditioned with MSC and M-OST.

SFM was conditioned with either undifferentiated MSC (MCM' used as control) or with M-OST (OCM) at distinct stages of maturation after 3-21 of osteogenic culture. The production of CM was accomplished by incubating SFM with MSC or M-OST overnight after which the supernatant was collected, centrifuged to remove large cell elements, aliquoted and then stored at -80°C without filtration. This procedure was previously shown in our lab to remove most M-OSTs since only traces of cells were present in OCM (≤ 200 cell/mL). Moreover, the total numbers of cells present in MSC and M-OST cultures used to produce the CM were found not to differ significantly (Table. 2).

Table 2. Total number of MSC and M-OST in cultures used to produce condition media

Conditions	Total Nucleated cell **number**
MSC CM	$6.35x10^5$
Day 6 OCM	$6.31x10^5$
Day 14 OCM	$6.38x10^5$
Day 21 OCM	$6.04x10^5$

Representative results from 2 MSC line shown, n=2.

MCM and OCMs were then used to culture $CD34^+$ cells isolated from pooled CB units. After 6 days of culture, CB cell expansion was measured by manual cell count or by cytometry and CB nucleated cells were analyzed by flow cytometry and by the progenitor assays CFC and LTC-IC. In order to avoid any donor-derived dominant effects, CMs were produced from 5 independent MSC lines and independent $CD34^+$ cell preparations (n=9) were used for the majority of the experiments presented in this thesis. The latter explain in part the large variation observed between independent experiments.

3. 2. 2 Expansion of $CD34^+$ HSPC in culture using OCM prepared with M-OST at distinct stages of maturation.

A preliminary investigation indicated that culture of CB $CD34^+$ cells in OCMs prepared with M-OST significantly promoted TNC expansion compared to control SFM. All OCM collected from M-OST differentiated past three days exhibited superior TNC expansion capability relative to the SFM control, which was statistically supported ($p<0.05$, Fig. 7). In contrast, TNC expansion was not significantly different in MCM cultures vs. SFM control (Fig. 7).

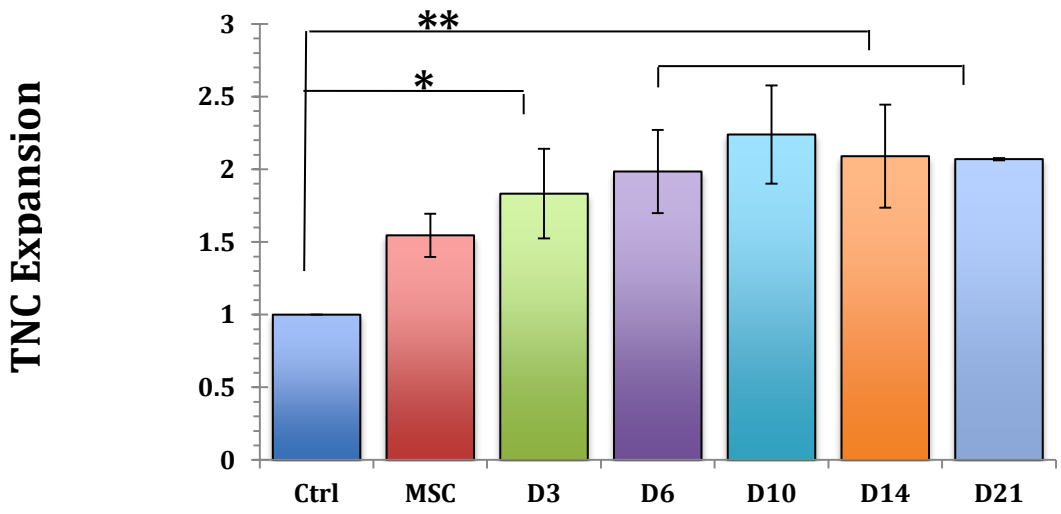
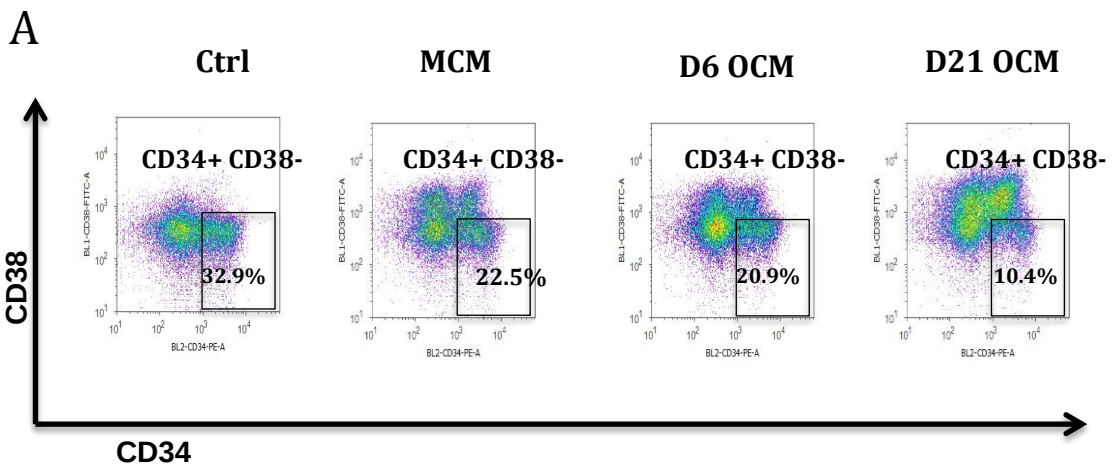


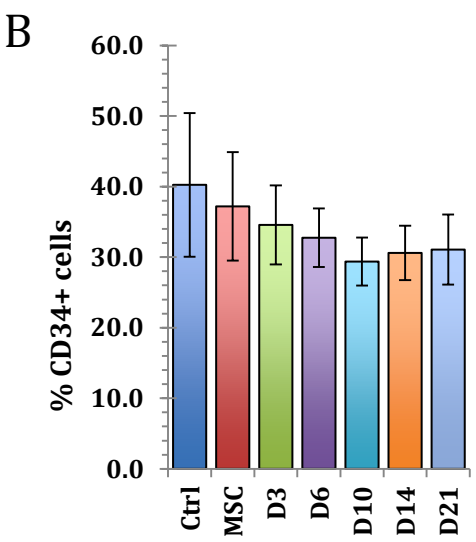
Figure 7. Expansion of CB TNC in control SFM (Ctrl), MCM, or in OCM prepared from M-OST at distinct stages of maturation (Day 3-21).

Data presented as TNC produced per starting CD34+ cells in CB cell cultures after six days of culture (mean ± SEM, n=3). Cultures done with CB CD34+ from independent donors and with MCM and OCM produced from independent MSC lines. Expansions of TNC were normalized to that obtained in the control SFM cultures. Significant differences determined by paired ANOVA test (Dunnet Multiple comparisons test). *$p<0.05$; **$p<0.01$ compared to Ctrl.

Also, I measured the expansion of $CD34^+$ cells by measuring the frequency of CD34+ cells and other cell subsets by flow cytometry (Fig. 8A). CD34 is an important marker for stem and progenitor cells and clinically this marker is used to predict engraftment potential of the graft (Geneugelijk & Spierings, 2015b; Kellner et al., 2015; Seita & Weissman, 2010). The frequencies of $CD34^+$ CB cells were similar in all culture conditions, ranging from 30-40% (Fig. 8B). However, the net output of $CD34^+$ cells tended to be at least greater (at least 1.5 - 2 fold) in all OCMs tested compared to control and MCM due to the elevated overall expansion with significant increase in day 3 and 21 OCM vs. SFM ($p<0.01$-0.05, Fig. 8C).

Next I investigated the impact of OCM on the expansion of $CD34^+CD38^-$ subpopulation (Fig 8A). This subpopulation is a rare cell fraction that is further enriched in HSCs and has the capacity to sustain engraftment for a long period of time (2-3 months)(Hao et al., 1995). Though differences were not all significant, there appeared to be an inverse correlation between the maturity of M-OST and the frequency and the net output of $CD34^+CD38^-$ cells since day 14 and 21 M-OST OCM produced the lowest frequency and production of $CD34^+CD38^-$ cells (Fig 8D, E).


A
Ctrl
MCM
D6 OCM
D21 OCM
CD34+ CD38-
32.9%
22.5%
20.9%
10.4%
CD38
CD34


B
% CD34+ cells
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Ctrl
MSC
D3
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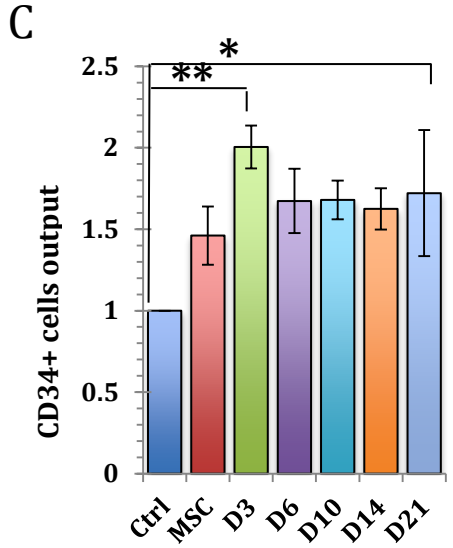
D10
D14
D21


C
CD34+ cells output
2.5
2
1.5
1
0.5
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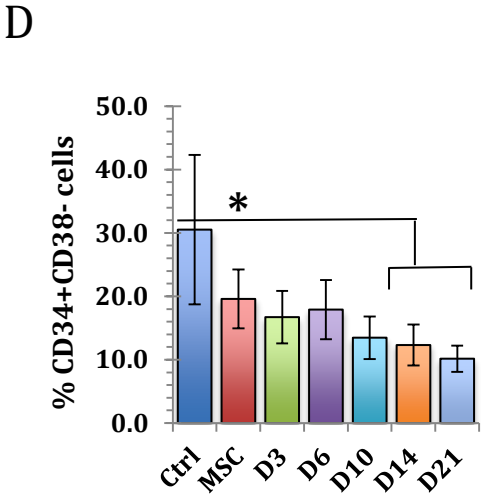
*
**
Ctrl
MSC
D3
D6
D10
D14
D21


D
% CD34+CD38- cells
50.0
40.0
30.0
20.0
10.0
0.0
*
Ctrl
MSC
D3
D6
D10
D14
D21

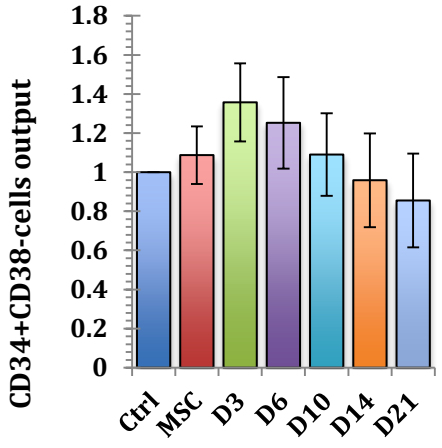

E
CD34+CD38-cells output
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ctrl
MSC
D3
D6
D10
D14
D21

Figure 8. Production of CB $CD34^+$ and $CD34^+CD38^-$ cells in control, MCM and OCM cultures.

A) Representative dot-plot flow cytometry analysis of CD34 and CD38 expression in CB cells from different cultures. Regions for $CD34^+CD38^-$ cells and frequencies are highlighted. B-C) Frequency of $CD34^+$ and production of $CD34^+$ cells per seeded cell. D-E) Frequency and production of $CD34^+CD38^-$ cells per seeded cell. Mean ± SEM of 3 independent experiments presented. Outputs of $CD34^+$ and $CD34^+CD38^-$ cells presented in C and E were normalized to that obtained in the control culture. Significant differences determined by Paired ANOVA test (Dunnet Multiple comparisons test). *$p<0.05$; **$p<0.01$ compared to Ctrl.

3. 2. 3 Impact of OCM on the expansion of CB CFC

To complement the previous phenotypic-based results with functional results, we monitored the expansion of committed progenitors using the CFC assay. Consistent with the rise in $CD34^+$ cell output, the net output of myeloid CFC progenitors was generally higher in all OCMs and MCM cultures vs. SFM control, though the differences were not all significant (Fig. 9). Also it can be observed that day 3, 6, 10, and 14 OCMs significantly increased the net output of the G/M/GM progenitors compare to SFM. Moreover and consistent with previous results, the net output or production of CFC was better with immature M-OST and lowest with mature M-OST produced after 14 and 21 days of osteogenic cultures, though differences were not quite significant (Fig 9).

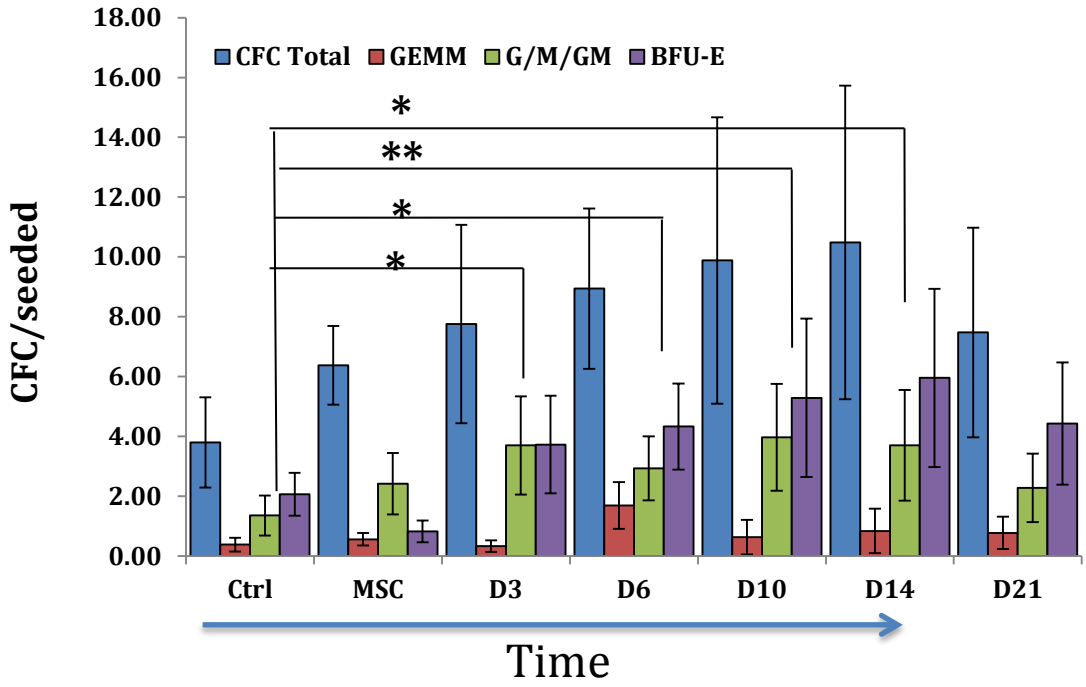


Figure 9. <u>Production of CFC in control, MSC CM and OCM cultures.</u>

Production of CFC produced per seeded cell obtained after six days of culture (mean ± SEM of 3 independent cultures). G/M/GM: Granulocyte, monocyte/macrophage, BFU-E: Erythroid, GEMM: Granulocyte, Erythroid, Macrophage, Megakaryocyte. Significant differences determined by paired ANOVA test (Dunnet Multiple comparisons test). *p<0.05; **p<0.01 compared to Ctrl.

3. 2. 4 Immature M-OST support greater expansion of immature CB cells enriched in HSPC

I set to further characterize the capacity of immature M-OST and mature M-OST to support the growth of CB $CD34^+$ cells. Day 6 and day 21 M-OST were selected as immature and mature M-OST respectively. As previously observed, OCM strongly promoted the expansion of TNC compared to MCM and SFM (Fig. 10A). Moreover, it was observed that day 6 and 21 OCMs produced the greatest increase in $CD34^+$ cell expansion relative to SFM ($p<0.05$, Fig. 10B). Again, differences between OCMs and MCM were not significant. Next, I examined the production of $CD34^+CD38^-$ subpopulation. As previously observed, production was highest with MCM and day 6 OCM though the differences vs. other cultures were not significant (Fig 10C). Also, there was a negative correlation between the maturity of M-OST and their impact on the production of $CD34^+CD38^-$ cells. Finally, the expansion of $CD34^+CD45RA^-$, another subpopulation enriched in stem and progenitors (Majeti, Park, & Weissman, 2007), tended to be increased in all cultures done with conditioned media compared to SFM control (Fig. 10D, $P>0.05$).

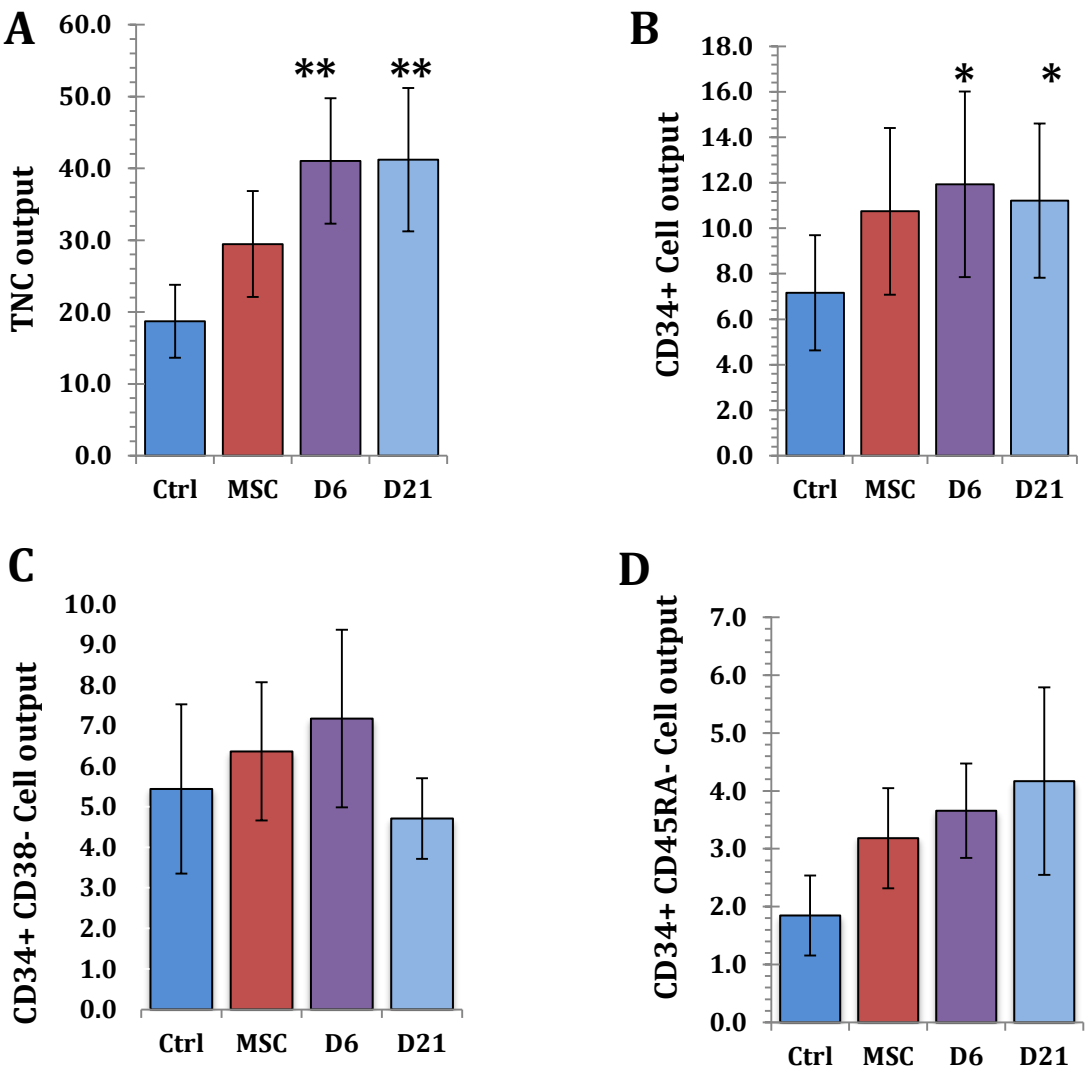
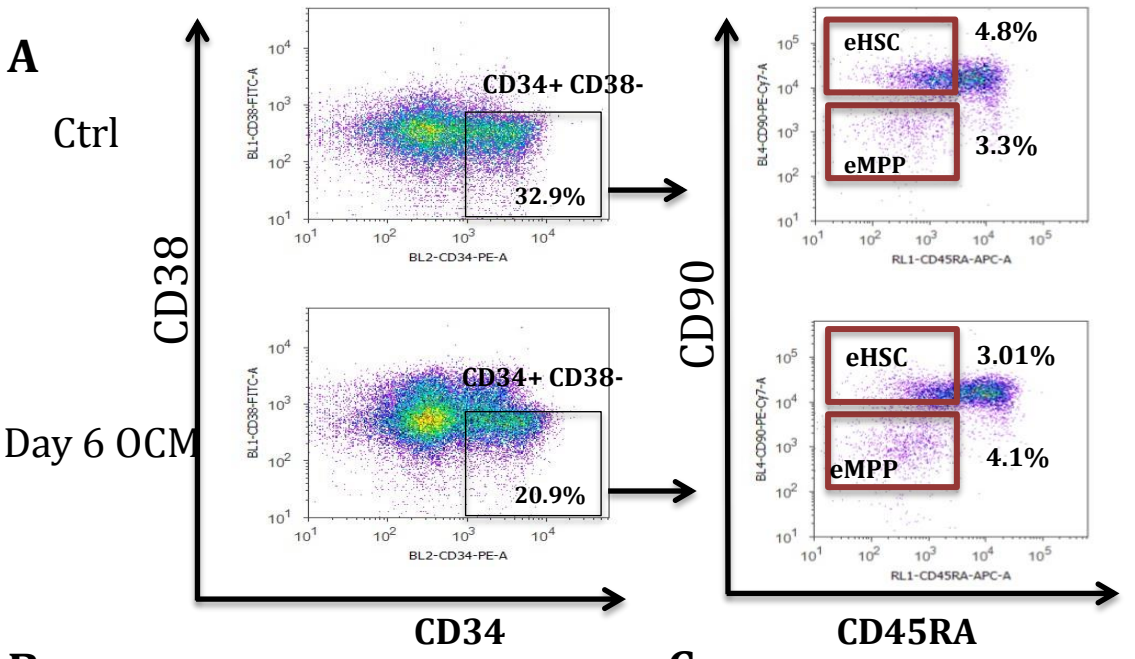


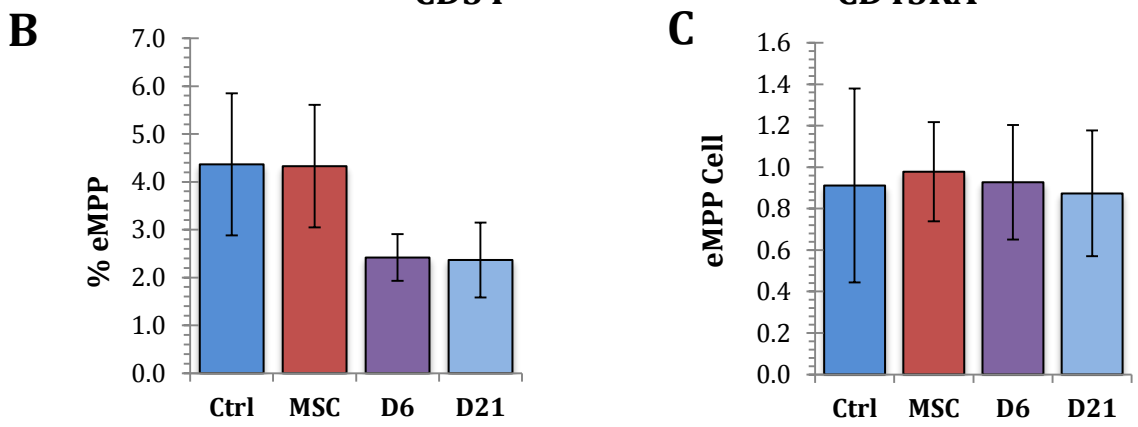
Figure 10. <u>Expansion of CB TNC and HSPC in control, MCM and OCM cultures</u>

Production per starting CD34+ cells in CB cell cultures after six days of culture. A) TNC expansion. B) Expansion of $CD34^+$ cells. C) Production of $CD34^+CD38^-$ cells. D) Production of $CD34^+CD45RA^-$ cells. Mean ± SEM of 6 independent cultures. Significant differences determined by Paired ANOVA test (Dunnet Multiple comparisons test).*$p< 0.05$;** $P< 0.01$ compared to Ctrl.

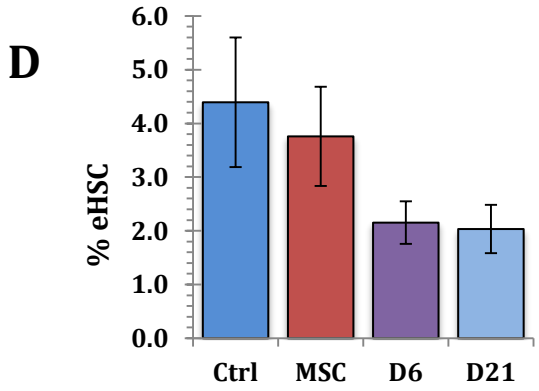
Following this, I tested the impact of OCM on MPP and LT-HSC subpopulation. HSC and progenitors can be tracked using a larger panel of antibodies against various cell surface antigen (Challen, Boles, Chambers, & Goodell, 2010). In flow cytometry analysis I first used CD34 and CD38 markers to obtain cells enriched in $CD34^{+}CD38^{-}$ subpopulation. Then this population was further assessed using CD90 and CD45RA markers for cells enriched in MPP and LT-HSC (Fig. 11A).

MPP are multipoint progenitors that do not possess long-term reconstitution activity (Challen et al., 2010). They have transient HSC activity and enriched in $CD34^{+}$ $CD38^{-}$ $CD45RA^{-}$ $CD90^{-}$ cell fraction (Majeti et al., 2007; Seita & Weissman, 2010). On the other hand, LT-HSC has long-term HSC activity (Challen et al., 2010). They are highly enriched in the $CD34^{+}$ $CD38^{-}$ $CD45RA^{-}$ $CD90^{+}$ cell fraction (Majeti et al., 2007). As shown in Fig. 11, similar results were obtained for the effect of the different media on the production of phenotypically like expanded (e) MPP and eLT-HSC; the frequencies of eMPP and eLT-HSC were generally reduced in OCM cultures vs. MCM and SFM (Fig. 11A, C) though the net production of either subpopulations was similar in all 4 conditions (Fig. 11B, D).


A
Ctrl
Day 6 OCM
CD38
CD34
CD90
CD45RA
CD34+ CD38-
32.9%
20.9%
eHSC
eMPP
4.8%
3.3%
3.01%
4.1%
BL1-CD38-FITC-A
BL2-CD34-PE-A
BL4-CD90-PE-Cy7-A
RL1-CD45RA-APC-A


B
% eMPP
Ctrl
MSC
D6
D21

C
eMPP Cell
Ctrl
MSC
D6
D21


D
% eHSC
Ctrl
MSC
D6
D21

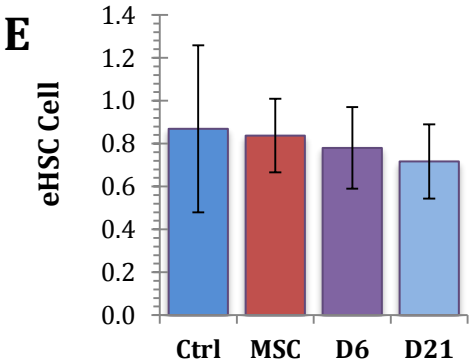

E
eHSC Cell
Ctrl
MSC
D6
D21

Figure 11. Expansion of CB HSPC in in control or OCM cultures

Production of phonotypical like expanded (e) eMPP and eHSC cells in cultures. A) Representative flow cytometry analysis of eMPP and eHSC enriched cell expression in cultures. B-C) Frequency and production of MPP per seeded cell. D-E) Frequency and production of eLT-HSC per seeded cell. Mean ± SEM of 6 independent cultures. No significant differences between the 4 conditions tested.

3. 2. 5 Impact of osteogenic maturation on the differentiation modulatory activities of OCMs on $CD34^+$ cells

Next, I investigated the impact of OCM on the differentiation and production of lineage committed CB cells. First, I checked the frequency of monocyte ($CD14^+$ cells) cells and demonstrated a gradual and significant increase with MCM, day 6 and day 21 OCM compared to SFM ($p<0.01$, Fig. 12A). Moreover, $CD14^+$ cell output showed also a gradual and significant increases with both OCMs compared to SFM ($p<0.01$, Fig. 12B). These results support are consistent with our previous findings (Dumont et al., 2014).

Afterward, I tested the impact of OCM on the frequency and production of megakaryocyte ($CD41^+$cells). It was observed that there were significant decreases in the frequency of $CD41^+$ cells with all OCMs and MCM compared to SFM ($p<0.01$, Fig. 12C). However, the net production of $CD41^+$ cells was not significantly different between the four cultures.

Finally, the frequency and production of erythrocytes ($CD235^+$ cells) was investigated (Fig. 12E, F). It can be observed that the frequency of $CD235^+$ cells was decreased in MCM and both OCMs, though not significantly. However, the production of $CD235^+$ cells tended to be increased in day 6 and 21 OCM compared to SFM ($P>0.05$), which also supports previous findings (Dumont et al., 2014).

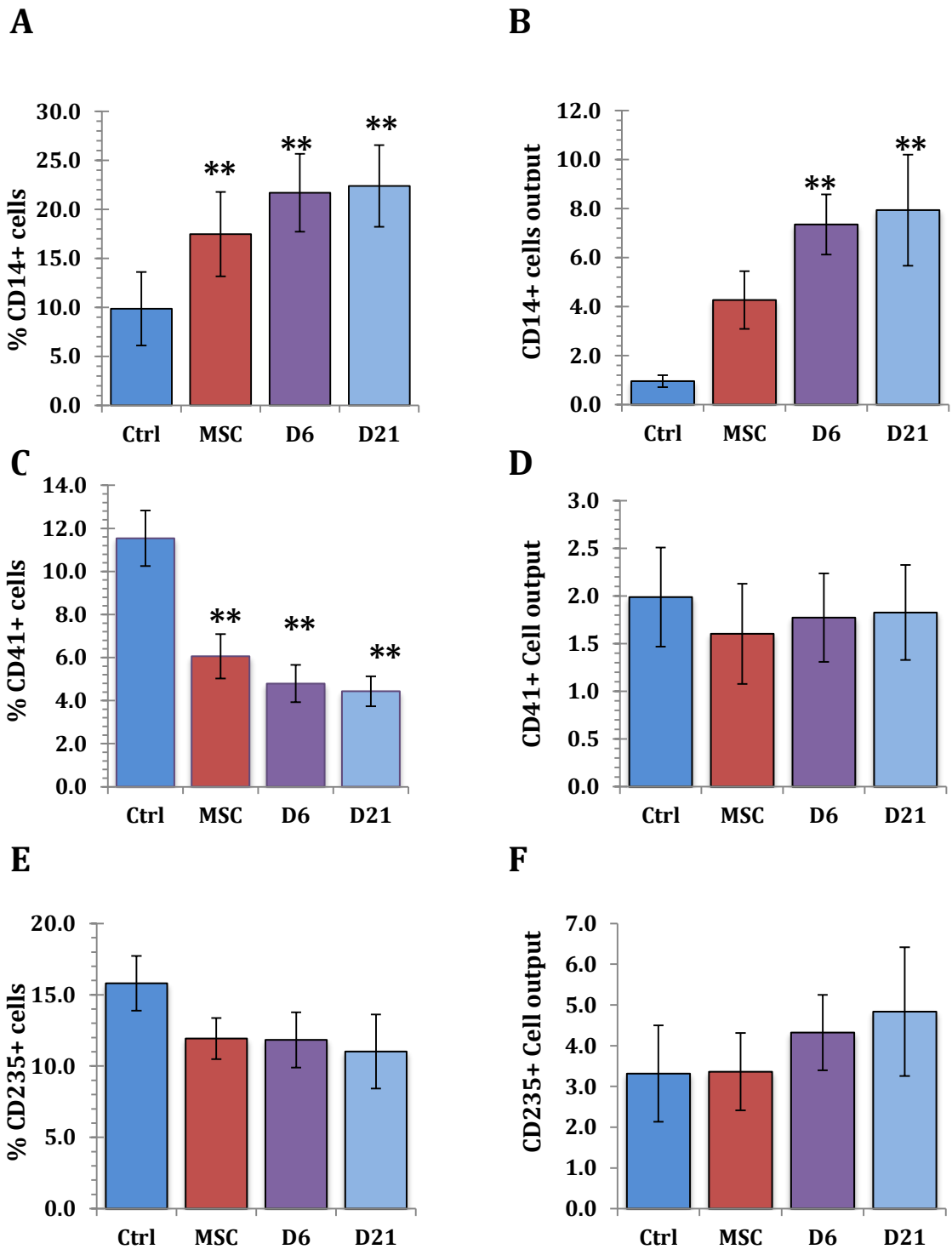

A
% CD14+ cells
30.0
25.0
20.0
15.0
10.0
5.0
0.0
**
**
**
Ctrl
MSC
D6
D21
B
CD14+ cells output
12.0
10.0
8.0
6.0
4.0
2.0
0.0
**
**
Ctrl
MSC
D6
D21
C
% CD41+ cells
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
**
**
**
Ctrl
MSC
D6
D21
D
CD41+ Cell output
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
MSC
D6
D21
E
% CD235+ cells
20.0
15.0
10.0
5.0
0.0
Ctrl
MSC
D6
D21
F
CD235+ Cell output
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Ctrl
MSC
D6
D21

Figure 12. Production of differentiated CB cells in cultures

CB CD34+ cells were cultured for 6 days in the different media. A-B) Frequency and production per seeded cell of $CD14^+$ cells. C-D) Frequency and production per seeded cell of $CD41^+$ cells. E-F) Frequency and production per seeded cell of $CD235^+$ cells. Mean ± SEM of 6 independent cultures. Significant differences determined by paired ANOVA (Dunnet Multiple comparisons test). *p< 0.05; **p<0.01 Compared to Ctrl.

3. 2. 6 Immature M-OST support greater expansions of CB CFC and multipotent progenitors

Next**,** I measured the impact of MCM, day 6 and day 21 OCMs on the expansion of myeloid progenitors using the CFC assay to further investigate whether day 6 OCM induced superior expansion of CFC. Only day 6 OCM significantly increased the production of total CFC ($p<0.01$, Fig. 13A) compared to SFM. While there was a trend of higher number of progenitors in day 6 OCM relative to MCM and day 21 OCM, the differences fail to be significant. Finally, production of CFC G/M/GM progenitors was most pronounced with day 6 OCM, followed by day 21 OCM and MCM (Fig. 13B).

Next, the LTC-IC assay was used to determine whether the conditioned media could increase the production of multipotent progenitors. This assay is used to measure progenitors with self-renewal and differentiation activities significantly superior to those detected in the CFC assay (Liu, Miller, & Eaves, 2013). A LDA was combined with LTC-IC to measure the frequency of the multipotent hematopoietic progenitors in the expanded cells. It can be observed that the frequency of LTC-IC in control SFM was superior to that seen in MCM and day 6 ($p<0.001$) and day 21 OCM ($p<0.001$, Fig. 14A). In addition, the frequency of LTC-IC in MCM was significantly higher than the frequency seen in day 6 and day 21 OCM ($p<0.05$, Fig. 14A). This is likely because there was significantly less cell growth in SFM (and to a lesser extent MCM) and therefore less differentiation pressure on the cycling UCB cells. More interestingly, the net output of LTC-IC produced per 10,000 starting $CD34^+$ cells was found to be highest in 3 out of 4 experiments with day 6 OCM of all

the media tested (Fig. 14 B-C). Conversely, the lowest production was observed with day 21 OCM (Fig. 14. B-C).

In summary, these results demonstrate that expansion of clonogenic and multipotent myeloid progenitors are generally greater in OCM produced from immature M-OST (i.e. day 6 M-OST) than the one produced with mature M-OST or MSC.

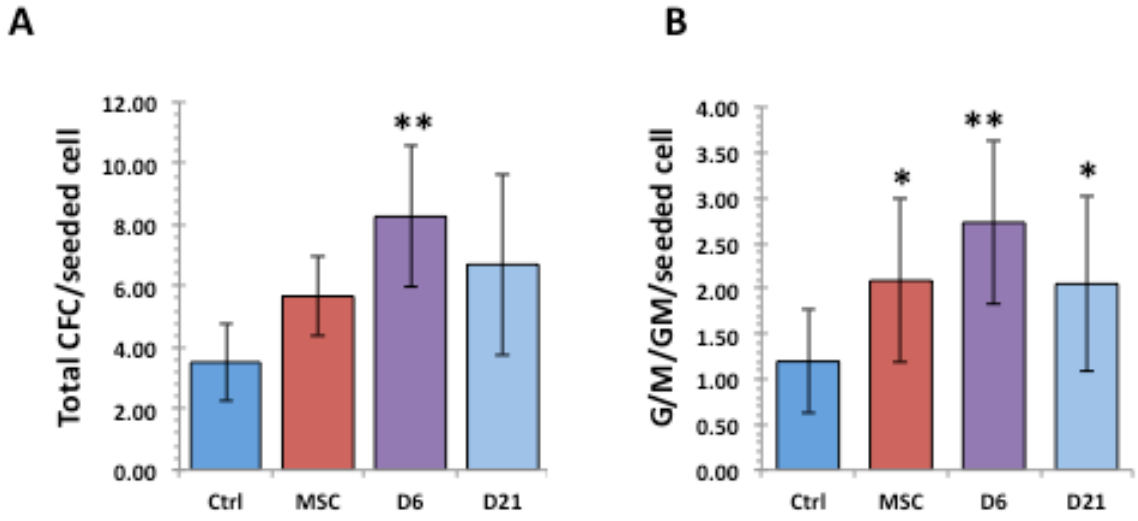


Figure 13. **Production of CFC in cultures**

Number of total (A) and G/M/GM (B) CFC progenitors produced per starting cells obtained after 6 days of culture (mean ± SEM of 6 independent cultures). Significant differences determined by paired ANOVA test (Dunnet Multiple comparisons test). *p< 0.05; **p<0.01 Compared to Ctrl.

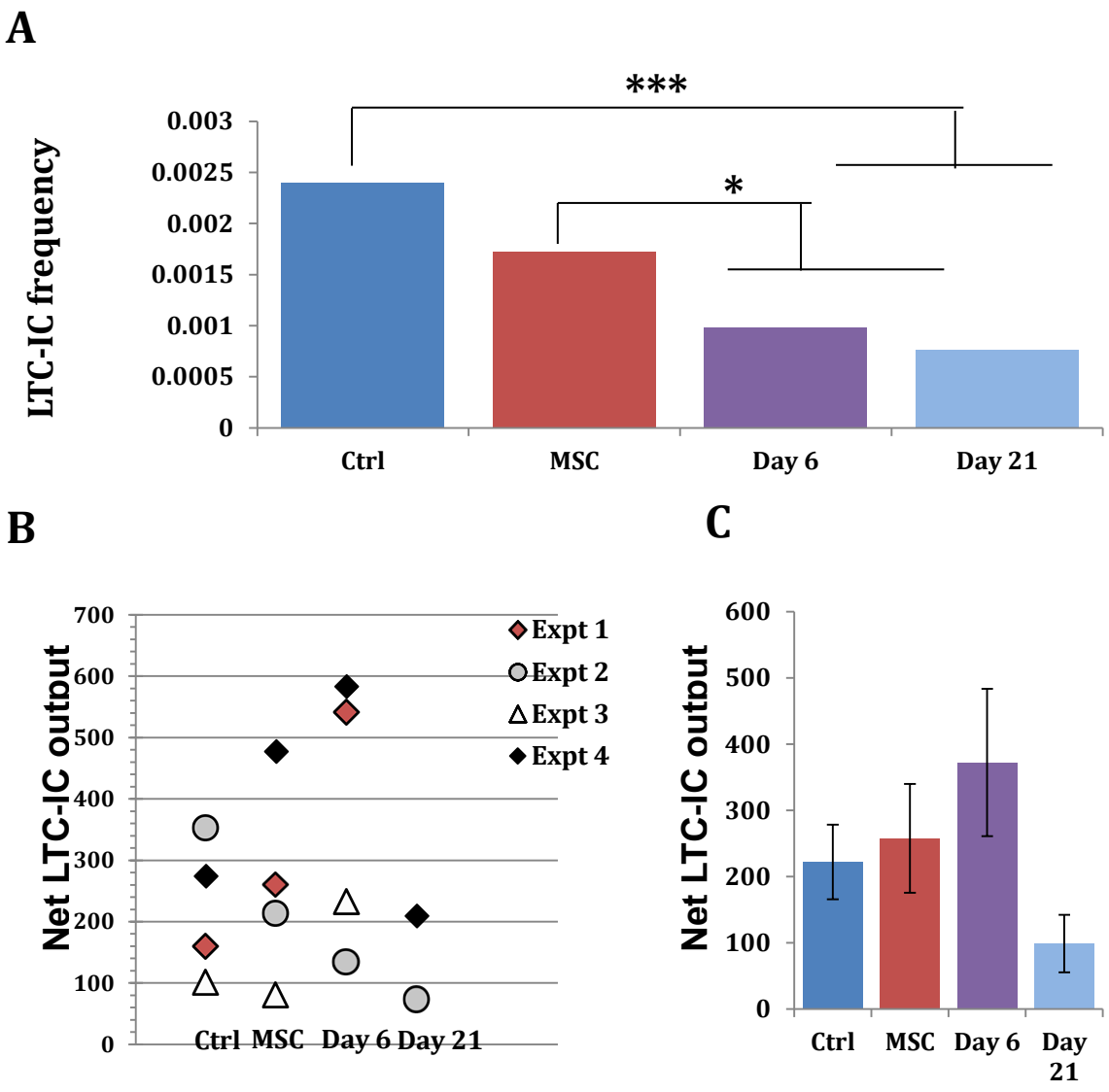


Figure 14. <u>Production of Long-Term Culture Initiating Cell LTC-IC:</u>

A) Frequency of LTC-IC measured in control, MCM and 6 OCM (mean ± SEM of 2-4 independent cultures) determined by LDA. The frequencies of LTC-IC cells in were determined using ELDA software. Significant differences determined by ELDA online software http://bioinf.wehi.edu.au/software/elda/ * $p<0.05$, ** $p<0.01$; ** $p<0.001$. B) Net output of LTC-IC produced per 10,000 starting CB CD34+ cell obtained in each culture (each symbol represent a different experiment (Expt)). C) Total number of LTC-IC produced per 10,000 starting CB CD34+ cell cultured for 6 days in control, MCM and day 6 OCM and day 21 OCM (mean ± SEM of 4 independent cultures except for day 21 OCM (n=2)).

3. 3 Investigating the role of Notch signally pathway in HSPC - supporting activity of OCM

3. 3. 1 Gene expression pattern of some Notch ligands in MSC and M-OST

OSTs are critical cell component of the hematopoietic stem cell niche. They are prominent for their enhancement of HSPC function and expansion through the up regulation of Notch signaling (Weber & Calvi, 2010). Calvi et al. (2003) found that OSTs produce high level of the Notch ligand Jagged-1, which participated in the activation of notch signaling and led to an increase in the growth and expansion of HSPCs *in vitro* (L. M. Calvi et al., 2003). Since low levels of M-OSTs (<200 M-OST/mL) remain in thawed OCM, we were interested to see how the Notch ligands are expressed in M-OST at different stages of differentiation. Whether the Notch pathway is instrumental in OCM capability to promote HSPC expansion was our next desired target to investigate.

First, the expression the Notch ligands *Jagged-1* and *-2, DLL-1* and *-4* was investigated in MSC undergoing osteogenic differentiation. qPCR analysis showed that the expression of *DLL-1* and *DLL-4* elevated with OST differentiation with significant increase in *DLL-4* ($p<0.05$, Fig. 15 C, D). In contrast, *Jagged-1* and *Jagged-2* expression were nearly equivalent in MSC and day 6 M-OST (Fig.15 A, B). These results confirm the expression of several transcripts for Notch ligands in M-OSTs, often with levels elevated to that of undifferentiated MSCs.

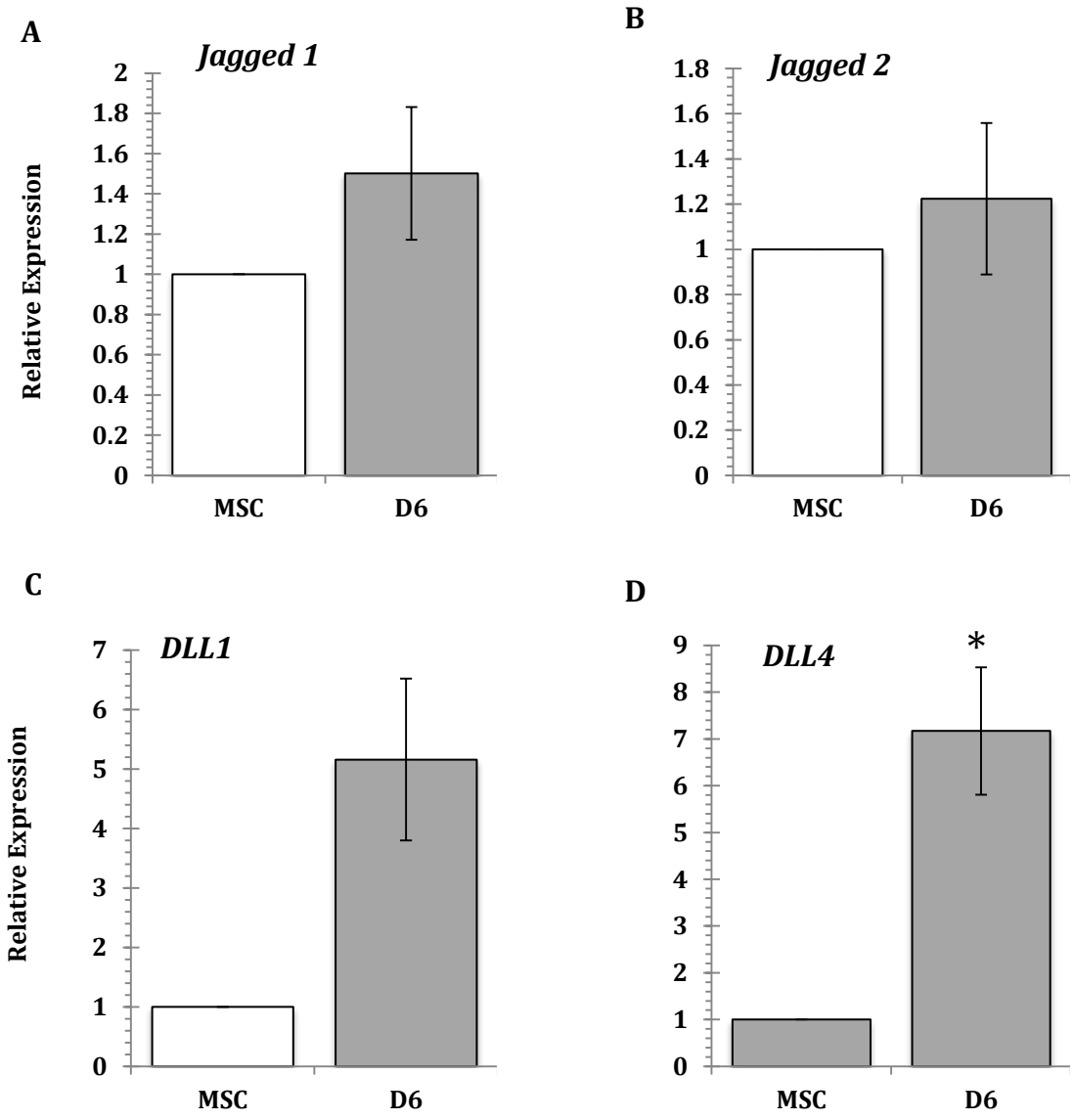


Figure 15. <u>Expression of Notch ligands in MSC and M-OST as a function of culture time</u>

Expression of Notch ligands in MSC and M-OSTs. Mean ± SEM of independent experiments, n=3 for *DLL4, Jagged-1* and *DLL1*, n=4 for *Jagged-2*. Relative expression normalised to GAPDH then evaluated against MSC expression levels. Significant differences determined paired t- test. *p< 0.05 compared to Ctr

3.3.2 γ Secretase inhibitor reduced the growth supporting activities in both SFM and OCM

To investigate whether notch pathway plays a role in the growth promoting activity of OCM, we cultured CB $CD34^+$ cultures in the presence or absence of the γ-secretase notch inhibitor (RO4929097). This compound effectively blocks notch activity by preventing notch cleavage (Saito et al., 2014). Dose-response experiments were performed in Jurkat cells to identify the dose that blocks Notch signaling in hematopoietic cells. We found that 2μM of RO4929097 was effective enough to reduce the expression of the Notch downstream targets *Hes-1* (80% reduction) and *Deltex* (90% reduction) (qPCR, data not shown).

Next, CB $CD34^+$ cultures were incubated with the inhibitor or with a vehicle control consisting of DMSO for comparison. The results showed that inhibition of Notch resulted in reduction of both TNC and $CD34^+$ cell expansion in control and in OCM cultures (Fig. 16). TNC and $CD34^+$ cell expansion was reduced similarly in both control and OCM cultures when 2 and 10 μM of the inhibitor was used compared to the DMSO control. When the reduction in both conditions was compared, it was observed that OCM still induced significantly higher expansion of TNC and CD34+ cells, even in the presence of the inhibitor. Therefore these results suggest that Notch pathway is unlikely to be an important mediator of OCM growth promoting activity on CB cells.

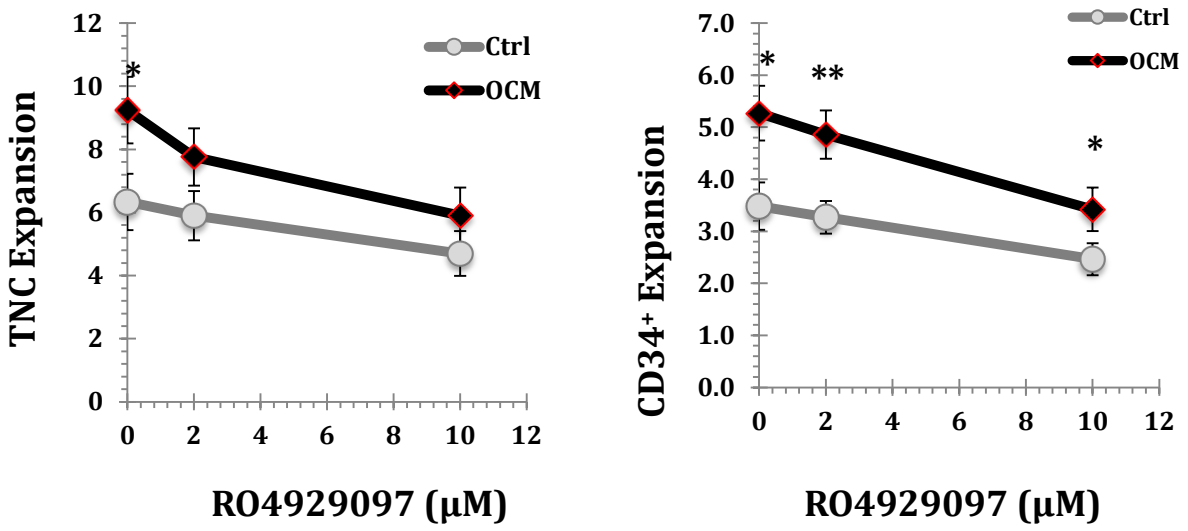


Figure 16. Impact of Notch inhibition on the growth of CB CD34⁺ cells in SFM and OCM cultures

Impact of γ secratase Notch-inhibitor RO4929097 on the production of TNC (left graph) and CD34⁺ cells per starting cell (right graph) in DMSO (Ctrl) and day 6 OCM cultures per starting cells in CB CD34⁺ cell. Cultures were treated with RO4929097 at day-0 and day-2 of culture (mean ± SEM of 5 independent cultures) and were analysed after four days to avoid potential toxicity of long treatment. Percentages of inhibition are shown. Significant differences determined by paired two-tailed T-test. *p< 0.05; **p<0.01 compared to DMSO Ctrl.

Next, I measured the impact of γ-secretase Notch inhibitor on the expansion of CB CFC in control and OCM cultures. In the control conditions it was observed that 10 μM of inhibitor reduced the numbers of colonies produced (G/M/GM, BFU-E, GEMM, and total CFC) compare to DMSO control (Fig. 17A). The same pattern of reduction was found in OCM (Fig. 17B). When comparing the SFM vs. OCM in the presence of Notch inhibitor, it was found that the number of G/M/GM and GEMM progenitors were still higher in OCM ($p<0.05$, data not shown). Also total CFC was higher in OCM than SFM ($P=0.1$, data not shown).

In summary, these results suggest that the Notch signaling pathway is not implicated in the OCMs increased growth promoting activities in supporting HSPC expansion.

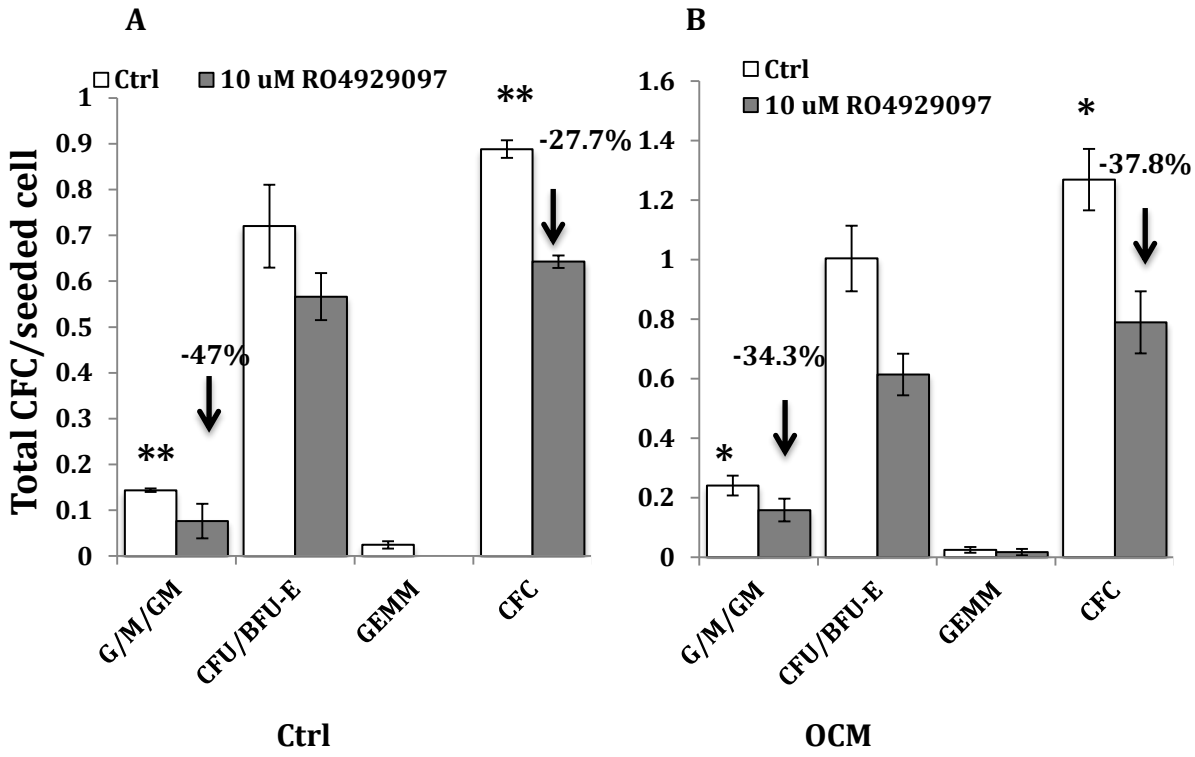


Figure 17. <u>Impact of Notch inhibition on the production of CFC in Ctrl or day 6 OCM cultures</u> Number of CFC produced per seeded cells in CB $CD34^+$ cell cultures after four days of culture in CB SFM (Ctrl) and OCM cultures treated with 10 uM of RO4929097 (mean ± SEM of 2 independent cultures). Significant differences determined by unpaired two-tailed T-test. *p<0.05; **p<0.01; ***p<0.001 compared to DMSO Ctrl.

3. 4 Gene expression pattern of selected regulators of HSPC in MSC and M-OST at distinct stages of maturation

Next, I measured by qPCR the transcript levels of selected GFs in MSC and M-OSTs. I focussed on IGFBP-2, and selected members of the Angpt-L family since these GFs can collaborate together to promote HSC expansion in SFM-based cultures (Walenda et al., 2011; Watts, Adair, & Kiem, 2011; Zhang, Kaba, Iizuka, Huynh, & Lodish, 2008)

The qPCR results revealed that the expressions of these GFs were generally higher in day6 M-OST compared to MSC with significant increase for Angpt-L2 and -5 ($p<0.05$, 0.001, Fig.18 A-D). Because of large variation between the different lines tested, no significant differences were observed for Angpt-L2 and -3 tested. However, as a whole these results suggest that the transcript levels of most *Angpt-L* genes are induced with osteogenic differentiation of MSC.

Finally, *IGFPB-2* expressions were also found to significantly increase with osteogenic differentiation observed in day 6 M-OST ($p< 0.05$, Fig. 18E). The increased level of *IGFPB-2* transcripts are consistent with our previous results that reported higher level of *IGFPB-2* in OCM vs. MCM (Dumont et al., 2014). In summary, these results show tendency of higher expression of several GFs that can promote growth of HSPC.

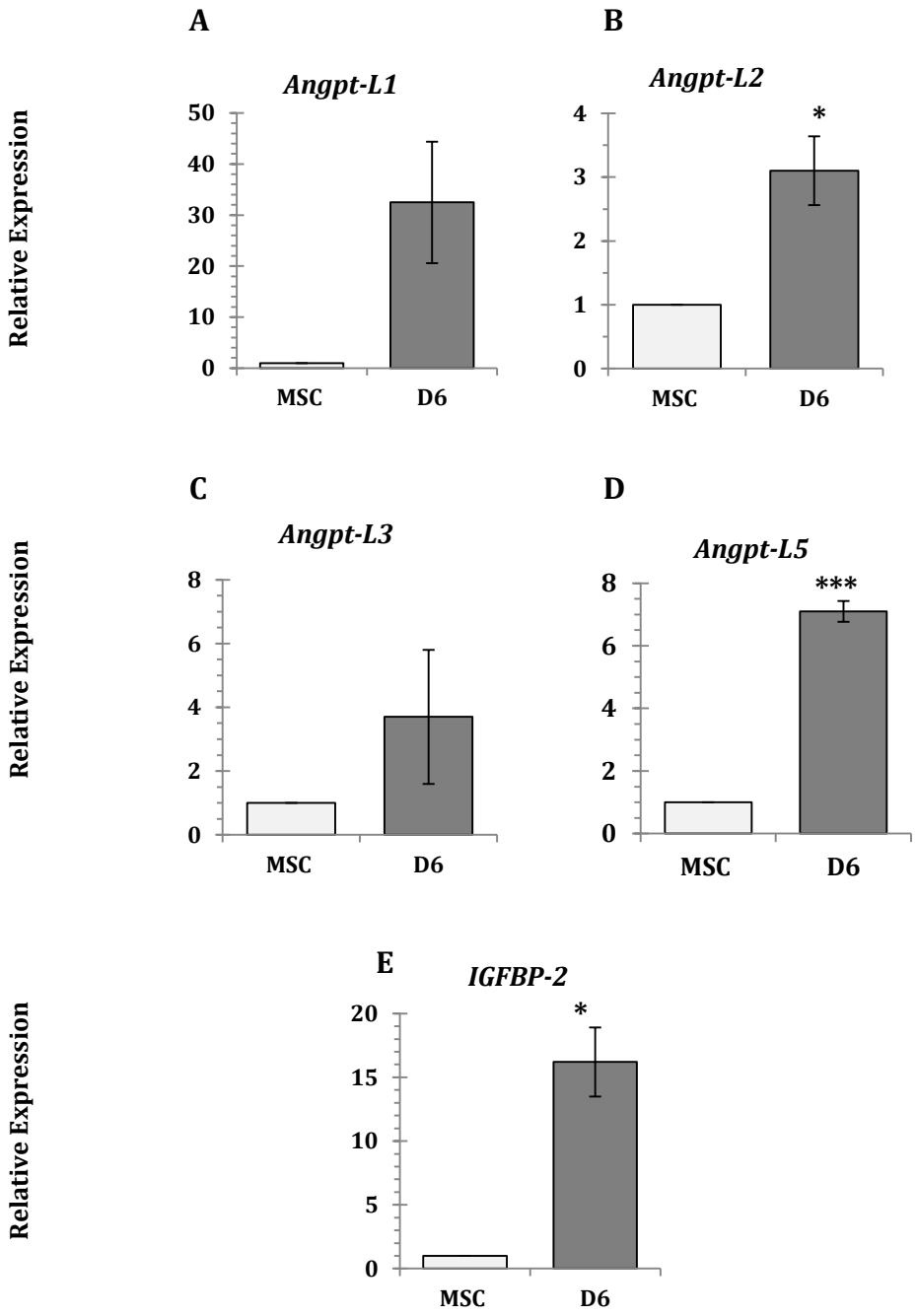


Figure 18. <u>Expression of *IGFBP-2* and *Angpt-L* members in MSC and M-OST</u>

Expression of *AngptL -1,-2, -3* and *-5*, and *IGFBP-2* transcript levels in M-OST relative to MSC. Expression normalised to GAPDH then evaluated against the expression level found in MSC. Mean ± SEM of 3 independent experiments. Significant differences determined by paired by paired t- test.*p< 0.05; ***p<0.001compared to Ctrl.

CHAPTER 4 DISCUSSION

Low doses of HSPCs in CB unit is partially responsible for the delays in platelet and neutrophil recoveries, which is considered a fundamental limitation in their usage as a HSC graft source. Many ex vivo expansion practices to increase the dose of stem and progenitor cells available have been studied to overcome this drawback, such as using stromal components in a way to mimic the normal BM environment (L. M. Calvi et al., 2003; de Lima et al., 2012; Salati et al., 2013; Taichman & Emerson, 1994; Taichman, Reilly, & Emerson, 1996). It has been previously reported by our group that OCM efficiently improved the platelet engraftment of ex vivo expanded CB $CD34^+$ cells. However, the mechanisms behind this action remain unknown.

Herein, I investigated if there was a link between the maturation status of M-OST and the capacity of OCM to promote production of CB cells and progenitors in cultures. M-OSTs were used as a model of human OSTs. The principal hypothesis of my thesis was that "**Hematopoietic supporting activity of M-OST would vary as a function of their maturation status**". To test this hypothesis, four objectives were set: 1) Produce M-OST at distinct stages of maturation. 2) Quantify the growth modulatory activities of OCM produced from these M-OSTs on CB $CD34^+$ cell cultures. 3) Test the Implication of Notch signaling in the mediation of OCM activity on CB CD34+ cell growth promotion 4) Compare the gene expression pattern of selected GFs of HSPC in MSC and M-OST.

4.1 Production of M-OST at distinct stages of maturation

I used M-OST to establish a model equivalent to primary OST to study distinct stages of osteogenic maturation. My results showed the progressive differentiation and maturation of M-OSTs over culture time. This was supported by the greater expressions of *Runx2* and *Osteocalcin* (Neve et al., 2011) and by the increase in Calcium deposits and ALP activity (Born et al., 2012; Chitteti, Cheng, Streicher, et al., 2010; Kulterer et al., 2007). These properties are typical criteria for OST that can identify them from other stromal cells. The ability of M-OSTs to express these genes, which are considered unique markers of OST, confirms their quality to be used as primary OST. Of note, I did not see significant differences in transcript levels for *RunX2* and *Osteocalcin* between different M-OST time points. This was unexpected but transcript levels and protein levels do not always correlate in MSC and M-OST, as recently reported (Foster et al,2005). In addition, the use of different human BM samples to establish that MSC likely play a role in the observed inter-experimental variation. However, the functional assays were consistent with M-OST undergoing progressive differentiation and maturation (Foster et al., 2005). Based on those results, I continued with my second objective.

4.2 Impact of M-OST maturation on the capcity of OCM to promote the growth of CB CD34+ and production of progenitors

The main goal of my project was to characterize the effects of OCM as a function of the M-OST differentiation and maturation status on the expansion of CD34+ cells and progenitors ex vivo. This investigation could provide insights into

the regulation of human HSC and progenitors by OST, and perhaps identify OCM with greater growth regulatory modulation on CB CD34+ cells. Moreover, this could provide an effective new option for a cellular therapy to improve double CB transplantation in the context of ex vivo expansion to promote and accelerate hematopoietic recovery or engraftment.

I investigated the capacity of OCMs (from day 3-21 M-OST) to promote the growth of CB $CD34^+$ cells in ex vivo culture sitting. This portion of my study demonstrated that all OCMs tested induced significantly greater TNC expansion than MCM and SFM. Conversely, TNC expansion with MCM was in between that induced by SFM and OCM. Moreover, the expansion of CD34+ was also generally superior with all OCM tested though differences vs. the SFM control were not always significant. These results suggest that the capacity of M-OST to promote the growth of CB cells and CD34+ cells is largely conserved from immature M-OST to mature M-OST. The lack of difference may be due to the fact that the M-OST may not undergo as much osteogenic maturation ex vivo than primary OSTs (Aubin, 2001; Lin & Hankenson, 2011). It will be interesting in future studies to compare the growth regulatory activity of OCM produced from M-OST versus primary OST to test this possibility. Furthermore, large differences between the different CB cultures are due to the use of independent CB CD34+ and M-OST cell preparations. These differences reduce the odds of finding significant differences although the trends were largely reproduced from culture to cultures.

Conversely, I saw a significant decrease in the proportion of $CD34^+CD38^-$ cells, an HSC enriched subset, in day 14 and day 21 OCM versus SFM control. Consistent with this; there was a trend of reduced production of $CD34^+CD38^-$ cells

with OCM produced from mature M-OST (>10 days) versus premature and immature M-OST (day 3-6 M-OST). Similar results were observed in the effect of OCMs on the net production of committed myeloid progenitors.

Based on these results and the redundant impact of OCMs, I focused my work on selected OCM produced from immature and mature M-OST (day 6 and day 21 M-OST respectively). Further analysis confirmed the previous observation that OCM better supports the production of TNC and CD34+ cells than SFM of MCM, and also provided further support that expansion of immature UCB subsets enriched in progenitors including CD34+CD38-, CFC and LTC-IC were mostly superior with OCM derived with day 6 M-OST rather than day 21 M-OST. Similar findings were reported by Chitteti el at. (2011), when they co-cultured LSK cells with primary murine OSTs. They found greater expansion of immature murine HSPC was associated with immature OSTs that have high expression of *Runx2*, low ALP activity and low calcium deposits, contrary to mature OSTs associated with elevated ALP activity and calcium deposits. Consequently our studies suggest that the growth promoting activity of M-OSTs on HSPC seems preserved between both species.

Additionally, my results also provide clear evidence that OCM do not solely support the expansion of primitive UCB subsets since the frequency, but not overall production, of CD34+ cells, CD34+CD38- cells, eMPP, eLT-HSC and LTC-IC cells were actually reduced in OCM cultures compared to MCM or SFM. Indeed, my data showed increases in the frequency and output of monocytes ($CD14^+$) and production of erythrocytes ($CD235^+$) in OCM cultures. These findings are supported by previous literature where increased monocyte and erythrocyte or just monocyte production

was found after HSC expanded with OST in a non-contact or contact coculture, respectively (Dumont et al., 2014; Salati et al., 2013). This data demonstrates that OCM promotes both the growth and differentiation of CB HSPC. The balance between self-renewal and differentiation of HSPCs is important for continuation of HSPC pool production. Salati et al. (2013) reported increased production of TNC and CFUs from CD34+ cells cocultured with primary OST, which was also found in my project using OCM from M-OST. However, the contact coculture condition with OST was able to maintain a higher percentage of CD34+CD38- cells than OCM. Taken together with my findings, it seems the contact between CD34+ and OSTs might lead to improved maintenance of some primitive HSPC subsets. Interestingly, CD34+CD38- have been deemed important for CFU and LTC-IC assays, and may account for the elevated CFU outputs seen (Hao et al., 1995).

4.3 Implication of Notch signalling in the mediation of OCM activity on CB CD34+ cell growth promotion

Many studies indicated the importance of Notch signalling in enhancing the HSC maintenance and self-renewal (L. M. Calvi et al., 2003; Delaney et al., 2010; Maillard et al., 2008; Weber & Calvi, 2010). Chittiti el at.(2010) demonstrated that Notch signalling was implicated in the activity of primary OSTs to promote expansion of murine HSC-enriched cells ex vivo in contact cultures. By using a γ-secretase inhibitor RO4929097, they inhibited Notch signalling and found a significant reduction of CB TNC and CFC production (Chitteti, Cheng, Poteat, et al., 2010). OST were also found to modulate the HSC niche through Notch signalling, with

abrogation of primitive HSC growth achieved via γ-secretase inhibition as well (L. M. Calvi et al., 2003).

In this study, we evaluated whether the hematopoietic supporting activity of OCM is dependent on Notch signalling. Numerous studies reported the expression of notch ligands by primary OSTs that elevated the growth of HSPCs by the activation of Notch signalling (L. M. Calvi et al., 2003; Chitteti, Cheng, Poteat, et al., 2010; Weber & Calvi, 2010).These ligands are known to be important in supporting stem cells expansion *in culture* (Pajcini, Speck, & Pear, 2011). My qPCR results demonstrated the expression of many Notch ligands (*DLL-1, DLL-4* and *Jagged-2*) in MSC and day 6 M-OST, with a pronounced increase of *DLL-1* and *DLL-4* expression in M-OST. Treatment with the Notch inhibitor RO4929097 reduced TNC, CB $CD34^+$ and progeny generation of CFC in cultures, but not exclusively to the OCM. This failure to solely affect the OCM condition suggests that Notch signaling is present in the SFM condition as well and does not constitute a characteristic feature enabling the growth promotion by OCM. Thus concluding that OST are also capable of promoting expansion of CD34+ cells independently of contact or Notch signaling.

4.4 Implication of several growth factors in the mediation of OCM activity on CB CD34+ cell growth promotion

In this study, I finally determined whether the OCM hematopoietic supporting activity could be modulated by the expression by M-OST of some GFs including IGFBP-2, AngptL-1, -2, -3, and -5. As reviewed by Pineault & Abu-Khader (2015), IGFBP-2 and AngptL-1, -2, -3, -5 have been shown to modulate HSC expansion ex

vivo and with signs of synergy (Huynh et al., 2008; Zhang et al., 2006, 2008). My qPCR results indicated the expression of these GFs in day 6 M-OST which may have partially affected the growth promoting activity of OCM and could synergize as previous found. In support to this, Dumont et al. reported the expression of IGFPB-2 in M-OST and that has been shown to contribute to HSC self-renewal (Dumont et al., 2014) . Thus, it is possible that some of the growth promoting activity of OCM could be mediated by the combination of some of those factors. Confirmation of this hypothesis would require detecting the presence of GFs in the OCM sample, which could be possible using protein-based analysis (e.g. enzyme-linked immunosorbent assay, Western blots). This is an important verification, as there is not always a correlation between transcript levels and proteins levels due to translation efficiency, degradation, etc.

To further understand the mechanisms behind the HSC growth promotion of OCM and its ability to induce expansion, other factors need to be examined. An example is the implication of other biochemical signalling pathways such as Wnt signalling, a non-contact pathway mediated by secreted ligands which has been implicated in modulating hematopoiesis (Florian et al., 2013; Reya et al., 2003) and studied in the coculture setting in the Pineault lab (unpublished results). Also, soluble cytokines (exogenous cytokines) that promote the growth and expansion of HSPCs and down-stream lineages are another interesting factor. Many studies reported the importance of several cytokines and several extra cellular matrix proteins secreted by OSTs such as TPO, FL, granulocyte colony-stimulating factor (G-CSF), macrophage (M-CSF) in supporting HSC maintenance and expansion ex

vivo and in vivo (Dumont et al., 2014; Mishima et al., 2010; Taichman et al., 1996). As evidenced in these studies, significant increases in HSC expansion were noted in the presence of these cytokines. Thus it would be interesting to determine if they are prevalent in OCM as well. In regards to studying the GFs, individual neutralization of selected GFs tested in this work could be a possible way to verify whether they are a major contributor to the growth promoting activity of OCM. This can be tested ex vivo using neutralized antibodies, similar to the concept utilized by (Akel, Petrow-Sadowski, Laughlin, & Ruscetti, 2003), or silencing (knockdown) of selected GF by siRNA in M-OST, similar to the concept utilized by (Mishima et al., 2010). Further analysis into, the GF interactions and their possible synergistic promoting activity in OCM can be achieved by using multiple neutralizing antibodies at once. Alternatively, adhesion molecules may also be involved in the OCM hematopoietic promotion activity and need to be investigated, as homing is a significant limitation to CB transplants. Examples of adhesion molecules, reviewed in Yin & Li (2006), are the angiopoietin-1/Tie2 signalling and Ca-sensing receptor (Adams et al., 2006; Arai et al., 2004).

Conclusion

Altogether, these results indicated that the growth promoting activity of M-OST on HSPC ex vivo expansion was generally greater in OCM and partially dependent on the maturation status of M-OST. Together these results only partially support my original hypothesis which was that “the hematopoietic supporting activity of OCM will vary as a function of the M-OST differentiation and maturation status”. On one hand, the expansion of TNC and CD34+ was not impacted by the

maturation status of M-OST. However, there were few significant differences between immature and mature M-OST. The capacity of OCM to expand immature CB progenitors including CD34+CD38- cells and multipotent progenitors was partially dependent on their maturation status. Moreover, I found OCM activity was independent of notch signalling, and that there is a possibility for the presence of several hematopoiesis-relevant GFs (which may lead to a synergistic effect – a significantly interesting focus in the future). The expression of some GFs such as IGFBP-2, AngptL-2 and -5 could be partially behind the OCM growth promoting activity as there was a correlation between the expression of these GFs and the increased production of $CD34^+$ cells and progenitors during M-OST differentiation, but more experiment needed to confirm these findings.

REFERENCES

Adams, G. B., Chabner, K. T., Alley, I. R., Olson, D. P., Szczepiorkowski, Z. M., Poznansky, M. C., … Scadden, D. T. (2006). Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature*, *439*(7076), 599–603. http://doi.org/10.1038/nature04247

Ahmed, N., Khokher, M. A., & Hassan, H. T. (1999). Cytokine-induced expansion of human CD34+ stem/progenitor and CD34+CD41+ early megakaryocytic marrow cells cultured on normal osteoblasts. *Stem Cells (Dayton, Ohio)*, *17*(2), 92–99. http://doi.org/10.1002/stem.170092

Akel, S., Petrow-Sadowski, C., Laughlin, M. J., & Ruscetti, F. W. (2003). Neutralization of autocrine transforming growth factor-beta in human cord blood CD34(+)CD38(-)Lin(-) cells promotes stem-cell-factor-mediated erythropoietin-independent early erythroid progenitor development and reduces terminal differentiation. *Stem Cells (Dayton, Ohio)*, *21*(5), 557–567. http://doi.org/10.1634/stemcells.21-5-557

Aldenhoven, M., & Kurtzberg, J. (2015). Cord blood is the optimal graft source for the treatment of pediatric patients with lysosomal storage diseases: clinical outcomes and future directions. *Cytotherapy*, *17*(6), 765–774. http://doi.org/10.1016/j.jcyt.2015.03.609

Andersson, E. R., & Lendahl, U. (2014). Therapeutic modulation of Notch signalling — are we there yet? *Nature Reviews Drug Discovery*, *13*(5), 357–378. http://doi.org/10.1038/nrd4252

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., … Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell*, *118*(2), 149–161. http://doi.org/10.1016/j.cell.2004.07.004

Aubin, J. E. (2001). Regulation of osteoblast formation and function. *Reviews in Endocrine & Metabolic Disorders*, *2*(1), 81–94.

Ballen, K. K. (2005). New trends in umbilical cord blood transplantation. *Blood*, *105*(10), 3786–3792. http://doi.org/10.1182/blood-2004-10-4125

Barros, A. P. D. N. de, Takiya, C. M., Garzoni, L. R., Leal-Ferreira, M. L., Dutra, H. S., Chiarini, L. B., … Rossi, M. I. D. (2010). Osteoblasts and Bone Marrow Mesenchymal Stromal Cells Control Hematopoietic Stem Cell Migration and Proliferation in 3D In Vitro Model. *PLOS ONE*, *5*(2), e9093. http://doi.org/10.1371/journal.pone.0009093

Benveniste, P., Serra, P., Dervovic, D., Herer, E., Knowles, G., Mohtashami, M., & Zúñiga-Pflücker, J. C. (2014). Notch signals are required for in vitro but not in vivo maintenance of human hematopoietic stem cells and delay the appearance of multipotent progenitors. *Blood*, *123*(8), 1167–1177. http://doi.org/10.1182/blood-2013-07-505099

Birmingham, E., Niebur, G. L., McHugh, P. E., Shaw, G., Barry, F. P., & McNamara, L. M. (2012). Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. *European Cells & Materials*, *23*, 13–27.

Born, A.-K., Lischer, S., & Maniura-Weber, K. (2012). Watching osteogenesis: life monitoring of osteogenic differentiation using an osteocalcin reporter. *Journal of Cellular Biochemistry*, *113*(1), 313–321. http://doi.org/10.1002/jcb.23357

Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., … Scadden, D. T. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*, *425*(6960), 841–846. http://doi.org/10.1038/nature02040

Calvi, L. M., & Link, D. C. (2015). The hematopoietic stem cell niche in homeostasis and disease. *Blood*, *126*(22), 2443–2451. http://doi.org/10.1182/blood-2015-07-533588

Celebi, B., Mantovani, D., & Pineault, N. (2011). Irradiated Mesenchymal Stem Cells improve the ex vivo expansion of Hematopoietic Progenitors by partly mimicking the bone marrow endosteal environment. *Journal of Immunological Methods*, *370*(1–2), 93–103. http://doi.org/10.1016/j.jim.2011.06.006

Challen, G. A., Boles, N. C., Chambers, S. M., & Goodell, M. A. (2010). Distinct Hematopoietic Stem Cell Subtypes Are Differentially Regulated by TGFβ1. *Cell Stem Cell*, *6*(3), 265–278. http://doi.org/10.1016/j.stem.2010.02.002

Cheng, Y.-H., Chitteti, B. R., Streicher, D. A., Morgan, J. A., Rodriguez-Rodriguez, S., Carlesso, N., … Kacena, M. A. (2011). Impact of maturational status on the ability of osteoblasts to enhance the hematopoietic function of stem and progenitor cells. *Journal of Bone and Mineral Research*, *26*(5), 1111–1121. http://doi.org/10.1002/jbmr.302

Chitteti, B. R., Cheng, Y.-H., Poteat, B., Rodriguez-Rodriguez, S., Goebel, W. S., Carlesso, N., … Srour, E. F. (2010). Impact of interactions of cellular components of the

bone marrow microenvironment on hematopoietic stem and progenitor cell function. *Blood*, *115*(16), 3239–3248. http://doi.org/10.1182/blood-2009-09-246173

Chitteti, B. R., Cheng, Y.-H., Streicher, D. A., Rodriguez-Rodriguez, S., Carlesso, N., Srour, E. F., & Kacena, M. A. (2010). Osteoblast lineage cells expressing high levels of Runx2 enhance hematopoietic progenitor cell proliferation and function. *Journal of Cellular Biochemistry*, *111*(2), 284–294. http://doi.org/10.1002/jcb.22694

Dazzi, F., Ramasamy, R., Glennie, S., Jones, S. P., & Roberts, I. (2006). The role of mesenchymal stem cells in haemopoiesis. *Blood Reviews*, *20*(3), 161–171. http://doi.org/10.1016/j.blre.2005.11.002

de Lima, M., McNiece, I., Robinson, S. N., Munsell, M., Eapen, M., Horowitz, M., … Shpall, E. J. (2012). Cord-Blood Engraftment with Ex Vivo Mesenchymal-Cell Coculture. *New England Journal of Medicine*, *367*(24), 2305–2315. http://doi.org/10.1056/NEJMoa1207285

Delaney, C., Heimfeld, S., Brashem-Stein, C., Voorhies, H., Manger, R. L., & Bernstein, I. D. (2010). Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nature Medicine*, *16*(2), 232–236. http://doi.org/10.1038/nm.2080

Deng, Z.-L., Sharff, K. A., Tang, N., Song, W.-X., Luo, J., Luo, X., … He, T.-C. (2008). Regulation of osteogenic differentiation during skeletal development. *Frontiers in Bioscience: A Journal and Virtual Library*, *13*, 2001–2021.

Devine, S. M., & Hoffman, R. (2000). Role of mesenchymal stem cells in hematopoietic stem cell transplantation. *Current Opinion in Hematology*, *7*(6), 358–363.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. ., Krause, D. S., … Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, *8*(4), 315–317. http://doi.org/10.1080/14653240600855905

Doulatov, S., Notta, F., Laurenti, E., & Dick, J. E. (2012). Hematopoiesis: a human perspective. *Cell Stem Cell*, *10*(2), 120–136. http://doi.org/10.1016/j.stem.2012.01.006

Dumont, N., Boyer, L., Émond, H., Çelebi-Saltik, B., Pasha, R., Bazin, R., … Pineault, N. (2014). Medium conditioned with mesenchymal stromal cell–derived osteoblasts improves the expansion and engraftment properties of cord blood progenitors. *Experimental Hematology*, *42*(9), 741–752.e1. http://doi.org/10.1016/j.exphem.2014.04.009

Fei, X. M., Wu, Y. J., Chang, Z., Miao, K. R., Tang, Y. H., Zhou, X. Y., … Wang, C. Y. (2007). Co-culture of cord blood CD34(+) cells with human BM mesenchymal stromal cells enhances short-term engraftment of cord blood cells in NOD/SCID mice. *Cytotherapy*, *9*(4), 338–347. http://doi.org/10.1080/14653240701291638

Florian, M. C., Nattamai, K. J., Dörr, K., Marka, G., Überle, B., Vas, V., … Geiger, H. (2013). A canonical to non-canonical Wnt signalling switch in haematopoietic stem-cell ageing. *Nature*, *503*(7476), 392–396. http://doi.org/10.1038/nature12631

Foster, L. J., Zeemann, P. A., Li, C., Mann, M., Jensen, O. N., & Kassem, M. (2005). Differential expression profiling of membrane proteins by quantitative proteomics in a human mesenchymal stem cell line undergoing osteoblast

differentiation. *Stem Cells (Dayton, Ohio)*, *23*(9), 1367–1377. http://doi.org/10.1634/stemcells.2004-0372

Friedman, M. S., Long, M. W., & Hankenson, K. D. (2006). Osteogenic differentiation of human mesenchymal stem cells is regulated by bone morphogenetic protein-6. *Journal of Cellular Biochemistry*, *98*(3), 538–554. http://doi.org/10.1002/jcb.20719

Geneugelijk, K., & Spierings, E. (2015a). Immunogenetic factors in the selection of cord blood units for transplantation: current search strategies and future perspectives. *Cytotherapy*, *17*(6), 702–710. http://doi.org/10.1016/j.jcyt.2015.03.002

Geneugelijk, K., & Spierings, E. (2015b). Immunogenetic factors in the selection of cord blood units for transplantation: current search strategies and future perspectives. *Cytotherapy*, *17*(6), 702–710. http://doi.org/10.1016/j.jcyt.2015.03.002

Gluckman, E., Broxmeyer, H. A., Auerbach, A. D., Friedman, H. S., Douglas, G. W., Devergie, A., … Lehn, P. (1989). Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *The New England Journal of Medicine*, *321*(17), 1174–1178. http://doi.org/10.1056/NEJM198910263211707

Golub, E. E., & Boesze-Battaglia, K. (2007). The role of alkaline phosphatase in mineralization: *Current Opinion in Orthopaedics*, *18*(5), 444–448. http://doi.org/10.1097/BCO.0b013e3282630851

Hao, Q. L., Shah, A. J., Thiemann, F. T., Smogorzewska, E. M., & Crooks, G. M. (1995). A functional comparison of CD34 + CD38- cells in cord blood and bone marrow. *Blood*, *86*(10), 3745–3753.

Hidalgo, A., & Frenette, P. S. (2005). Enforced fucosylation of neonatal CD34+ cells generates selectin ligands that enhance the initial interactions with microvessels but not homing to bone marrow. *Blood*, *105*(2), 567–575. http://doi.org/10.1182/blood-2004-03-1026

Horwitz, M. E., Chao, N. J., Rizzieri, D. A., Long, G. D., Sullivan, K. M., Gasparetto, C., … Peled, T. (2014a). Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. *Journal of Clinical Investigation*, *124*(7), 3121–3128. http://doi.org/10.1172/JCI74556

Horwitz, M. E., Chao, N. J., Rizzieri, D. A., Long, G. D., Sullivan, K. M., Gasparetto, C., … Peled, T. (2014b). Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. *The Journal of Clinical Investigation*, *124*(7), 3121–3128. http://doi.org/10.1172/JCI74556

Horwitz, M. E., & Frassoni, F. (2015). Improving the outcome of umbilical cord blood transplantation through ex vivo expansion or graft manipulation. *Cytotherapy*, *17*(6), 730–738. http://doi.org/10.1016/j.jcyt.2015.02.004

Hu, Y., & Smyth, G. K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *Journal of Immunological Methods*, *347*(1–2), 70–78. http://doi.org/10.1016/j.jim.2009.06.008

Huang, W., Yang, S., Shao, J., & Li, Y.-P. (2007). Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Frontiers in Bioscience: A Journal and Virtual Library*, *12*, 3068–3092.

Huynh, H., Iizuka, S., Kaba, M., Kirak, O., Zheng, J., Lodish, H. F., & Zhang, C. C. (2008). Insulin-Like Growth Factor-Binding Protein 2 Secreted by a Tumorigenic Cell Line Supports Ex Vivo Expansion of Mouse Hematopoietic Stem Cells. *STEM CELLS*, *26*(6), 1628–1635. http://doi.org/10.1634/stemcells.2008-0064

Jaiswal, N., Haynesworth, S. E., Caplan, A. I., & Bruder, S. P. (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *Journal of Cellular Biochemistry*, *64*(2), 295–312.

Jung, Y., Wang, J., Havens, A., Sun, Y., Wang, J., Jin, T., & Taichman, R. S. (2005). Cell-to-cell contact is critical for the survival of hematopoietic progenitor cells on osteoblasts. *Cytokine*, *32*(3–4), 155–162. http://doi.org/10.1016/j.cyto.2005.09.001

Kellner, J., Li, S., Zweidler-McKay, P. A., Shpall, E. J., & McNiece, I. (2015). Phenotypic and functional comparison of mobilized peripheral blood versus umbilical cord blood megakaryocyte populations. *Cytotherapy*, *17*(4), 418–427. http://doi.org/10.1016/j.jcyt.2014.11.004

Kulterer, B., Friedl, G., Jandrositz, A., Sanchez-Cabo, F., Prokesch, A., Paar, C., … Trajanoski, Z. (2007). Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics*, *8*, 70. http://doi.org/10.1186/1471-2164-8-70

Larionov, A., Krause, A., & Miller, W. (2005). A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics*, *6*, 62. http://doi.org/10.1186/1471-2105-6-62

Le Blanc, K., & Mougiakakos, D. (2012). Multipotent mesenchymal stromal cells and the innate immune system. *Nature Reviews Immunology*, *12*(5), 383–396. http://doi.org/10.1038/nri3209

Lin, G. L., & Hankenson, K. D. (2011). Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. *Journal of Cellular Biochemistry*, *112*(12), 3491–3501. http://doi.org/10.1002/jcb.23287

Liu, M., Miller, C. L., & Eaves, C. J. (2013). Human long-term culture initiating cell assay. *Methods in Molecular Biology (Clifton, N.J.)*, *946*, 241–256. http://doi.org/10.1007/978-1-62703-128-8_15

Maillard, I., Koch, U., Dumortier, A., Shestova, O., Xu, L., Sai, H., … Pear, W. S. (2008). Canonical Notch Signaling Is Dispensable for the Maintenance of Adult Hematopoietic Stem Cells. *Cell Stem Cell*, *2*(4), 356–366. http://doi.org/10.1016/j.stem.2008.02.011

Majeti, R., Park, C. Y., & Weissman, I. L. (2007). Identification of a Hierarchy of Multipotent Hematopoietic Progenitors in Human Cord Blood. *Cell Stem Cell*, *1*(6), 635–645. http://doi.org/10.1016/j.stem.2007.10.001

Mendelson, A., & Frenette, P. S. (2014). Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nature Medicine*, *20*(8), 833–846. http://doi.org/10.1038/nm.3647

Mishima, S., Nagai, A., Abdullah, S., Matsuda, C., Taketani, T., Kumakura, S., … Masuda, J. (2010). Effective ex vivo expansion of hematopoietic stem cells using osteoblast-differentiated mesenchymal stem cells is CXCL12 dependent. *European Journal of Haematology*, *84*(6), 538–546. http://doi.org/10.1111/j.1600-0609.2010.01419.x

Neve, A., Corrado, A., & Cantatore, F. P. (2011). Osteoblast physiology in normal and pathological conditions. *Cell and Tissue Research*, *343*(2), 289–302. http://doi.org/10.1007/s00441-010-1086-1

Pajcini, K. V., Speck, N. A., & Pear, W. S. (2011). Notch signaling in mammalian hematopoietic stem cells. *Leukemia*, *25*(10), 1525–1532. http://doi.org/10.1038/leu.2011.127

Pineault, N., Cortin, V., Boyer, L., Garnier, A., Robert, A., Thérien, C., & Roy, D.-C. (2011). Individual and synergistic cytokine effects controlling the expansion of cord blood CD34(+) cells and megakaryocyte progenitors in culture. *Cytotherapy*, *13*(4), 467–480. http://doi.org/10.3109/14653249.2010.530651

Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., … Weissman, I. L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*, *423*(6938), 409–414. http://doi.org/10.1038/nature01593

Riddell, J., Gazit, R., Garrison, B. S., Guo, G., Saadatpour, A., Mandal, P. K., … Rossi, D. J. (2014). Reprogramming committed murine blood cells to induced hematopoietic stem cells with defined factors. *Cell*, *157*(3), 549–564. http://doi.org/10.1016/j.cell.2014.04.006

Rocha, V., Wagner, J. E., Sobocinski, K. A., Klein, J. P., Zhang, M.-J., Horowitz, M. M., & Gluckman, E. (2000). Graft-Versus-Host Disease in Children Who Have Received a Cord-Blood or Bone Marrow Transplant from an HLA-Identical Sibling. *New England Journal of Medicine*, *342*(25), 1846–1854. http://doi.org/10.1056/NEJM200006223422501

Sabokbar, A., Millett, P. J., Myer, B., & Rushton, N. (1994). A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. *Bone and Mineral*, *27*(1), 57–67. http://doi.org/10.1016/S0169-6009(08)80187-0

Saito, N., Fu, J., Zheng, S., Yao, J., Wang, S., Liu, D. D., … Koul, D. (2014). A high Notch pathway activation predicts response to γ secretase inhibitors in proneural subtype of glioma tumor initiating cells. *Stem Cells (Dayton, Ohio)*, *32*(1), 301–312. http://doi.org/10.1002/stem.1528

Salati, S., Lisignoli, G., Manferdini, C., Pennucci, V., Zini, R., Bianchi, E., … Manfredini, R. (2013). Co-culture of hematopoietic stem/progenitor cells with human osteblasts favours mono/macrophage differentiation at the expense of the erythroid lineage. *PloS One*, *8*(1), e53496. http://doi.org/10.1371/journal.pone.0053496

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*, *4*(1–2), 7–25.

Seita, J., & Weissman, I. L. (2010). Hematopoietic Stem Cell: Self-renewal versus Differentiation. *Wiley Interdisciplinary Reviews. Systems Biology and Medicine*, *2*(6), 640–653. http://doi.org/10.1002/wsbm.86

Sharpless, N. E., & DePinho, R. A. (2007). How stem cells age and why this makes us grow old. *Nature Reviews Molecular Cell Biology*, *8*(9), 703–713. http://doi.org/10.1038/nrm2241

Shiozawa, Y., Havens, A. M., Pienta, K. J., & Taichman, R. S. (2008). The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. *Leukemia*, *22*(5), 941–950. http://doi.org/10.1038/leu.2008.48

Stanford, C. M., Jacobson, P. A., Eanes, E. D., Lembke, L. A., & Midura, R. J. (1995). Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). *The Journal of Biological Chemistry*, *270*(16), 9420–9428.

Summers, C., Milano, F., Gooley, T. A., Dahlberg, A., & Delaney, C. (2014). Infusion of Ex Vivo Expanded Cord Blood Progenitor Cells Reduces the Risk of Bacteremia after Myeloablative Cord Blood Transplant. *Blood*, *124*(21), 3860–3860.

Suresh, S., & Irvine, A. E. (2015). The NOTCH signaling pathway in normal and malignant blood cell production. *Journal of Cell Communication and Signaling*, *9*(1), 5–13. http://doi.org/10.1007/s12079-015-0271-0

Taichman, R. S., & Emerson, S. G. (1994). Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. *The Journal of Experimental Medicine*, *179*(5), 1677–1682.

Taichman, R. S., Reilly, M. J., & Emerson, S. G. (1996). Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. *Blood*, *87*(2), 518–524.

Tse, W., & Laughlin, M. J. (2005). Umbilical cord blood transplantation: a new alternative option. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*, 377–383. http://doi.org/10.1182/asheducation-2005.1.377

Wagner, J. E., Brunstein, C. G., Boitano, A. E., DeFor, T. E., McKenna, D., Sumstad, D., … Bleul, C. C. (2016). Phase I/II Trial of StemRegenin-1 Expanded Umbilical Cord Blood Hematopoietic Stem Cells Supports Testing as a Stand-Alone Graft. *Cell Stem Cell*, *18*(1), 144–155. http://doi.org/10.1016/j.stem.2015.10.004

Walenda, T., Bokermann, G., Ventura Ferreira, M. S., Piroth, D. M., Hieronymus, T., Neuss, S., … Wagner, W. (2011). Synergistic effects of growth factors and mesenchymal stromal cells for expansion of hematopoietic stem and progenitor cells. *Experimental Hematology*, *39*(6), 617–628. http://doi.org/10.1016/j.exphem.2011.02.011

Watts, K. L., Adair, J., & Kiem, H.-P. (2011). Hematopoietic stem cell expansion and gene therapy. *Cytotherapy*, *13*(10), 1164–1171. http://doi.org/10.3109/14653249.2011.620748

Weber, J. M., & Calvi, L. M. (2010). Notch Signaling and the Bone Marrow Hematopoietic Stem Cell Niche. *Bone*, *46*(2), 281–285. http://doi.org/10.1016/j.bone.2009.08.007

Zhang, C. C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., & Lodish, H. F. (2006). Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. *Nature Medicine*, *12*(2), 240–245. http://doi.org/10.1038/nm1342

Zhang, C. C., Kaba, M., Iizuka, S., Huynh, H., & Lodish, H. F. (2008). Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood*, *111*(7), 3415–3423. http://doi.org/10.1182/blood-2007-11-122119

Zhu, F., Friedman, M. S., Luo, W., Woolf, P., & Hankenson, K. D. (2012). The transcription factor osterix (SP7) regulates BMP6-induced human osteoblast differentiation. *Journal of Cellular Physiology*, *227*(6), 2677–2685. http://doi.org/10.1002/jcp.23010